Oestrogen and the post-myocardial infarction heart: benefit or detriment?

Helen L Jeanes

PhD-The University of Edinburgh-2006

Declaration

I hereby declare that all the work described in this thesis was performed entirely by myself, except for the procedures stated in the acknowledgements. The work contains no material that has been accepted for the award of any other degree or diploma in any university or tertiary institution and to the best of my knowledge contains no material published or written by any other person, except where stated in the text.

Helen Lindsey Jeanes

Acknowledgements

There are countless people I would like to thank for their help, support and guidance throughout the course of my PhD. Below are some of those who's contribution has been so great that they can not escape without being named.

Firstly I would to thank my supervisors, Dr Gillian Gray and Dr Simon Riley for their continued guidance and support throughout. Thank you also to Professor Adriano Rossi for his commitment to my thesis committee. Within the lab Isam and Rose have played enormous roles, between them teaching me just about all the surgical techniques and experimental procedures that I have used within the laboratory and Shelia in histology was very helpful when the immuno techniques were not working. The lab would not have been the same and such a pleasurable place to work had it not been for everyone else there, including Fiona, Catriona, Sarah, Mike, Katie, Katrina, Mark, Phil, Graema, Danny, Ross, Aime and Nicki.

I would like to thank and acknowledge the work of Grace Grant who carried out all the processing of tissue into wax blocks, Rosemary Leask and Ellen Bruce who carried out all the zymography experiments, Ian Swanston who carried out the oestrogen radioimmunoassays and Agnieszka Kozak who did the immunohistochemistry.

Besides those in the lab, I would like to acknowledge the support I have had from Andy over the last year while writing. Especially for the long runs nad bicycle rides which were a great break and distraction.

Not least, thanks to my parents for all their continued support, without which I would not be where I am now. Along with them Roz and Yvonne have been fabulous. Nicola, Evie and Tom have also been a great help providing lots of fun times.

Abstract

Oestrogen (E) is thought to be protective within the cardiovascular system. Pre-menopausal women have a lower risk of developing cardiovascular disease than age-matched men but this difference is lost at the menopause. E initially protects the myocardium following myocardial infarction (MI), yet there is evidence that pre-menopausal women have a higher acute mortality rate following MI, an effect that is also seen in experimental MI. Recent clinical trials show that hormonal replacement therapy is not effective for prevention of cardiovascular disease in post-menopausal women.

This thesis aimed firstly to investigate the effects of E on the myocardium during healing after MI. In ovariectomised female mice, E treatment results in an increase in acute mortality due to cardiac rupture following induction of MI by coronary artery ligation. We hypothesised that E modifies the remodelling process, particularly through regulation of matrix metalloproteinase (MMP) enzyme activity. After MI, there was a reduction in necrotic myocardial tissue and inflammatory response in E treated compared to placebo treated mice. However there was no concomitant reduction in MMP-2 and MMP-9 activity. E treatment was also associated with a reduction in MMP-13 expression and an increase in tissue inhibitor of metalloproteinase type 2 activity. These data show that E disrupts matrix degradation during infarct healing after MI. In the mouse this results in an increased incidence of cardiac rupture.

A second aim of the thesis was to determine the roles of the E receptor (ER) α and ERβ in mediating the initial cardioprotective effects of E following MI. E reduced infarct size, neutrophil infiltration and generation of oxidant stress following myocardial ischaemia with reperfusion in rats. This effect was not blocked by a novel ERβ antagonist but was mimicked by a novel ERα agonist. The ERα agonist also protected the isolated buffer perfused heart

from reperfusion injury showing that it has both neutrophil dependent and independent effects.

A third aim of the thesis was to determine whether inclusion of a progesterone analogue in hormone replacement therapy (HRT) regimes modifies the cardioprotective effects of E. In ovariectomised female rats, E supplementation reduced infarct size and neutrophil infiltration following ishaemia and reperfusion compared to placebo treated animals. This protection was lost in animals receiving the progesterone analogue medroxyprogesterone acetate (MPA) in addition to E. Co-administration of MPA therefore negates the beneficial effects of E in myocardial reperfusion injury.

In conclusion, the data confirm the initial cardioprotective effects of E following myocardial infarction with or without reperfusion. At least in the rat, this protection is mediated through stimulation of the ERα, through direct effects on the heart and additional effects on neutrophils. This protective influence of E is lost if E is co-administered with MPA, as it frequently is in HRT regimes. In addition, despite the initial cardioprotection E can influence subsequent matrix remodelling during healing of the myocardial infarct through modification of MMP activity. In the mouse this destabilises the infarct and results in an increased incidence of cardiac rupture. E therefore has both beneficial and detrimental effects in the post-MI heart.

Presentations and Publications

Publications

Full Papers:

Jeanes, H.L. Wanikiat, P. Sharif, I. Gray, G.A. 'Medroxyprogesterone acetate inhibits the cardioprotective effect of estrogen in experimental ischaemia-reperfusion injury' (2006). *Menopause* **13** (1): 80-86. *See appendix 3.*

Sharif, I. Jeanes, H.L. Leask, R. Bruce, E. Webb, D.J. Riley, S.C. Gray, G.A. 'Secretion of matrix metalloproteinases-2 & -9 post-myocardial infarction, and rate of cardiac rupture, is increased in C57B6J/129sv compared to C57B6J mice' (2006). *Submitted to 'Laboratory Investigation'.*

Jeanes, H.L. Sharif, I. Kozak, A, Leask, R. Bruce, E. Webb, D.J Riley, S. Gray, G.A. '17β-estradiol increases the incidence of cardiac rupture post myocardial infarction despite suppression of cell death, neutrophil influx and matrix metalloproteinase activation.' (2006). *Submitted to 'Basic Research in Cardiology'.*

Jeanes, H.L. Tabor, C. Sharif, I. Webb, D.J Gray, G.A. 'ER alpha, but not ER beta, mediates the cardioprotective effects of oestrogen following ischaemia-reperfusion injury in the rat.' (2006). *Under internal review at Organon prior to submission to British Journal Pharmacolgy.*

Review: Jeanes, H.L. Newby, D.E. Gray, G.A. 'Cardiovascular risk in women: the impact of hormone replacement therapy and prospects for new therapeutic approaches' (2006). Accepted at: *Expert Opinion in Pharmacotherapy.*

Abstracts:

Jeanes, H.L. Wanikiat, P. Sharif, I. Gray, G.A. 'Medroyprogesterone acetate inhibits the cardioprotective effect of oestrogen after ischaemia reperfusion in the rat' (2004). *British Journal of Pharmacology*; Proceedings Supplement. **2** (2); 016P. *See appendix 3.*

Jeanes, H.L. Sharif, I. Kozak, A, Leask, R. Bruce, E. Riley, S. Gray, G.A. '17β-Estradiol modifies the profile of myocardial MMP and TIMP during healing post-myocardial infarction in mice' (2006). *Journal of Molecular and Cellular Cardiology*; (40); A84, 862-918. *See appendix 3.*

Presentations

Oral Presentations:

Annual Meeting for the British Pharmacological Society, University of Bath:
'Medroyprogesterone acetate inhibits the cardioprotective effect of oestrogen after ischaemia reperfusion in the rat'. 6th July 2004.

Scottish Cardiac Forum, Strathclyde University, Glasgow:
British Council-INYS workshop on Cellular and Molecular Aspects of Cardiac Function, Bratislava, Slovakia:
'17β-estradiol modifies the profile of MMP and TIMP activation during wound healing post-myocardial infarction in mice'. 4th February and 2nd March 2006.

Poster Presentations:

The Scottish Cardiac Forum, Glasgow:
'Medroxyprogesterone acetate inhibits the cardioprotective effect of oestrogen following ischemia and reperfusion in the rat heart'. 4th January 2004.

The 3rd James Black Conference, The British Pharmacological Society – Pharmacological Insights and Therapeutic Targets in heart Failure, Oxford:
'17β-Estradiol modifies the profile of myocardial MMP and TIMP activation during wound healing post-myocardial infarction in mice.' 19th September 2005.

The 28th International Society For Heart Research-Translating Molecular Advances. North American Section. Toronto, Canada:
'17β-Estradiol modifies the profile of myocardial MMP and TIMP during healing post-myocardial infarction in mice'. 14th June 2006

Abbreviations

Abbreviation	
$17\beta E_2$	17β estradiol
AAR	Area-at-risk
ACE	Angiotensin Converting Enzyme
AF	Activation Factor
ANOVA	Analysis of varience
APS	Ammonium persulphate
B	Border
bFGF	basic Fibroblast Growth Factor
BSA	Bovine serum albumin
CEE	Conjugated equine oestrogen
CHD	Coronary heart disease
CRP	C-reactive protein
CVD	Cardiovascular disease
Cys	Cysteine
DAB	3'3-diaminobenzidine
DPBS	Dulbecco's phosphate buffered saline
E	Oestrogen
ECL	Enhanced Chemiluminesence
ECM	Extracellular matrix
ELITE	Early versus Late Intervention Trial with Estradiol
eNOS	endothelial nitric oxide synthase
EPR	Electroparamagnetic resonance
ERα	Oestrogen receptor α
ERβ	Oestrogen receptor β
ERA	Oestrogen Replacement and Atherosclerosis
ERE	Oestrogen Response Element
ERT	Oestrogen replacement therapy
FGF-1	Fibroblast Growth Factor
GPI	glycosyl-phosphatidylinositol
HDL	High-density-lipoprotein
HERS	Heart and Estrogen/progesterone replacement study
HRP	Horseradish-peroxidase
HRS	Hours
HRT	Hormone replacement therapy
HSP	Heat Shock Protein
HTAB	Hexacyltrimethylammonium bromide
I	Infarct

Abbreviation	
i.p	Intraperitoneally
ICAM	Intercellular adhesion molecule
IL	Interleukin
KEEPS	Kronos Early Estradiol Protection Study
KO	Knock-out
LCA	Left Coronary Artery
LDL	Low-density-lipoprotein
LV	Left Ventricle
MABP	Mean arterial blood pressure
M-CSF	Macrophage Colony Stimulating Factor
MI	Myocardial Infarction
MMP	Matrix metalloproteinase
MOM	Mouse-on-mouse
MPA	Medroxyprogesterone acetate
MPO	Myeloperoxidase
MT-MMP	Membrane matrix metalloproteinase
nNOS	neuronal NOS
NO_2	Nitric dioxide
NO	Nitric oxide
NSB	Non-specific binding
OVX	Ovariectomy
PA	Plasminogen activator
PBS	Phosphate buffered saline
POF	Premature ovarian failure
ROS	Reactive oxygen species
RUTH	Raloxifene Use for The Heart
RV	Right Ventricle
S	Septum
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of mean
SOD	Superoxide dismutase
TBS	Tris buffered saline
TEMED	N,N,N,N'-tetramethylethylenediamine
TESPA	3-aminopropyltriethoxy-saline
TIMP	Tissue inhibitor of metalloproteinases
TNF-α	Tumor-necrosis-factor
t-PA	tissue-Plasminogen activator
TTBS	Tween tris buffered saline
TTC	2,3,5-Triphenyltetrazolim Chloride

Abbreviation	
TUNEL	Tat-mediated-dUTP Nick End Labelling
u-PA	urokinase-Plasminogen activator
VEGF	Vascular Endothelial Growth Factor
VSMC	vascular Smooth Muscle Cell
WHI	Womens Health Initiative
WISE	Womens Ischaemia Syndrome Evaluation

CONTENTS

Tables and Figures

Figures

CHAPTER 1

INTRODUCTION

1.1. Female gender and cardiovascular disease

Cardiovascular disease (CVD) including coronary heart disease (CHD) that encompasses heart failure, stroke and myocardial infarction (MI) is the leading single cause of mortality in the developed world. In 2004 216,379 deaths in the UK were due to CVD (Heartstats.org 2005). Current lifestyles within the western world do not indicate that this trend will abate soon, although over the next 5 years the British government aim to reduce the number of CVD related deaths in under 75 yr olds by 40%. With increasingly ageing populations the number of people at risk from CVD is also escalating, thus making it even more important for us to understand the mechanisms of CVD and develop strategies for its prevention and treatment (Herrington *et al.* 2003a). There is a general misconception that CVD is a disease predominantly effecting males. CVD including CHD, stroke and peripheral vascular disease is actually the number one killer of females. In 2004 in the UK CVD accounted for 36% of deaths in females compared to all cancers, which accounted for 24% of deaths (Figure 1.1).

A possible reason for this misconception is that the onset of CVD occurs on average 10 years later in females than in males (Lerner *et al.* 1986). In peri-menopausal women the incidence of CVD is 1 in 6, and lower than the incidence in age-matched men. After the menopause the incidence rate of CHD and CVD related mortality rises to 1 in 3 and matches the rate in males (Vaccarino *et al.* 1999). Figure 1.2 demonstrates that prior to the menopause (<45 years of age) males have a higher incidence of CVD but this gender difference is reversed after the menopause (55-64 years). Association of the menopause with an increased risk of CVD has resulted in the belief that oestrogen (E) protects females prior to the menopause. Plasma levels of E drop significantly during the menopause, which coincides with the increased incidence of CVD. The importance of E within CVD is supported by observations in younger women who have premature ovarian failure (POF). These women undergo premature menopause and consequently have lower E levels and are at an increased risk of mortality, especially from CVD including stroke and coronary artery disease (van der Schouw *et al.* 1996). These observations of young women with POF highlight the significant role E has within CVD

protection and demonstrate that the observations seen at the menopause are not only an age related occurrence.

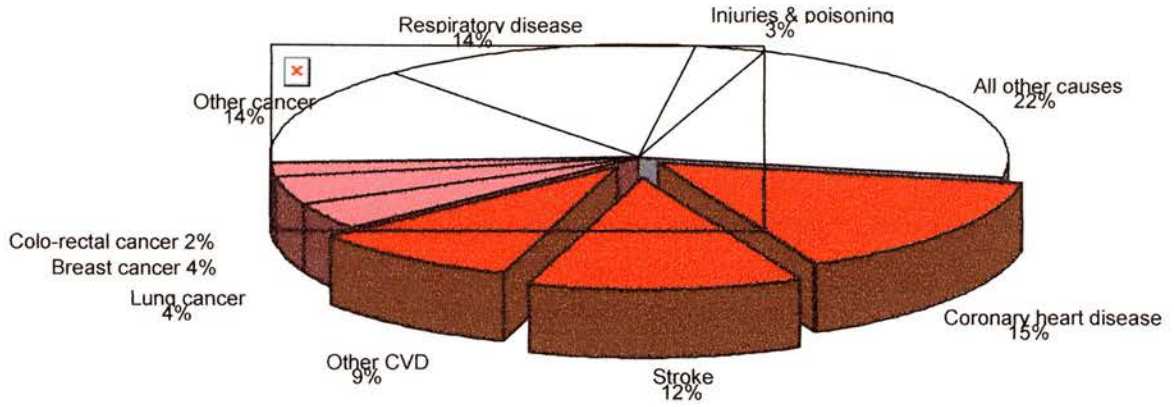


Figure 1.1. **Female mortality, grouped by cause in the UK.**
Data is from 2004, www.heartstats.org. Reprinted with permission from The British Heart Foundation

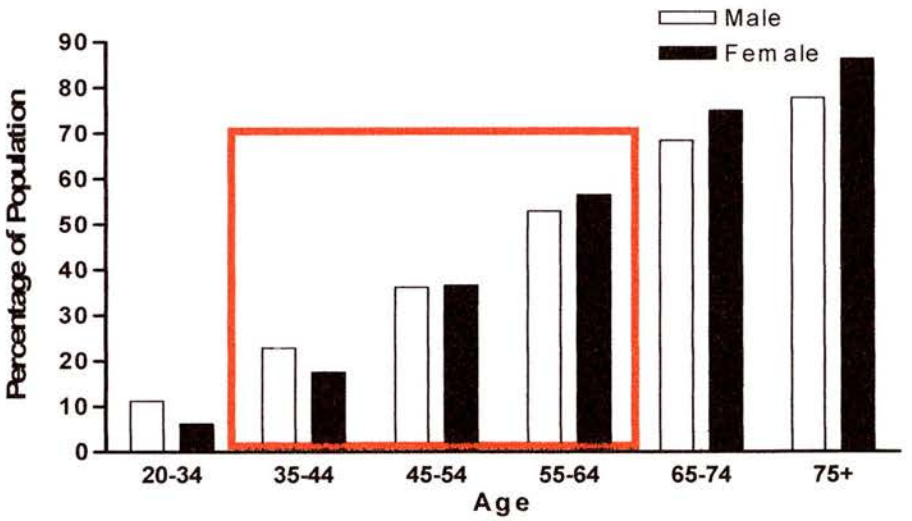


Figure 1.2. **The Prevalence of CVD**
The prevalence of CVD in Americans, grouped by age and sex. The data includes CHD, heart failure, stroke and hypertension in Americans. Data is from National Health and Nutrition Examination Survey (NHANES) 1999-2002, Centre for Disease/ national Centre for Health Science (CDC/NCHS).

1.2. Oestrogen synthesis and storage

There are three predominant naturally occurring Es in the human body, estradiol, estriol and estrone. Estradiol is the most abundant and efficacious E prior to the menopause, after which estrone becomes the most profuse. E is predominantly synthesised in the ovaries and corpus luteum, outside of the ovaries, lesser quantities are synthesised in the liver, adrenal glands, adipose tissue, breasts, blood vessels and heart (Dubey *et al.* 2004).

Characterised as a steroid hormone, the initial precursor of E synthesis is cholesterol, whilst the immediate precursor varies depending on the E. Estradiol is synthesised from testosterone, whilst estrone from androstenedione, these two Es inter-convert through the action of 17β-hydroxysteroid dehydrogenase (Figure 1.3). The plasma concentrations of E are relatively constant in men, whilst in women the concentration varies depending on the phase of the estrous cycle. Post-menopause the plasma level is greatly reduced (O'Shaughnessy *et al.* 2001; Table 1.1). Synthesis of Es outside the ovaries means the plasma level of E is not always an accurate measure of the tissue levels.

	17β-estradiol plasma concentration/100ml
Males	0.8 ng
Follicular phase	5-35 ng
Luteal phase	10-20 ng
Post-menopause	1-2 ng

***Table 1.1.* Human 17β-estradiol plasma concentrations.**
Concentration of 17β estradiol in males and at different phases of the female oestrous cycle and post-menopause (Knobil and Neill 1988).

Metabolism of E occurs in the liver through the action of 17β-hydroxysteroid dehydrogenase, or in the kidney to water-soluble compounds, such as catecholestradiols (Figure 1.3). A wide range of physiological processes are influenced by E, including the

growth and differentiation of reproductive cells, brain and bone development as well as roles in regulation of blood vessel diameter, and hence blood flow, and pathologically for instance in breast cancer (Erlandsson *et al.* 2005; McEwan 2002; Seeger *et al.* 2005). This project is focused upon the cardiovascular effects of E and therefore the other systemic properties of E will not be discussed further.

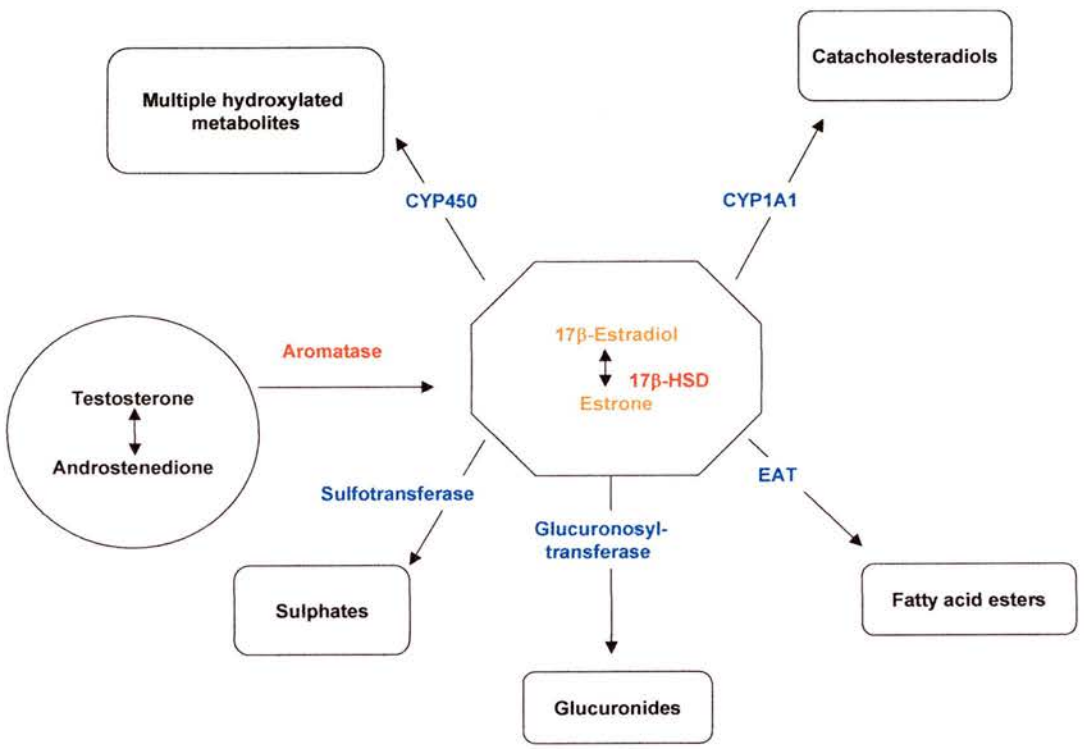


Figure 1.3. **Oestrogen synthesis and degradation pathways.**
(Adapted from Dubey *et al.* 2004).

1.2.1. Oestrogen receptors

E diffuses freely in and out of cells. High affinity binding to specific ligand activated receptors retains E in cells and mediates its effects. The majority of Es actions are through it acting upon classical steroid receptors, these classical genomic effects are observed hours and days after the administration of E. Currently there are two known E receptor (ER) subtypes, ERα and ERβ.

1.2.1.1. Classical oestrogen receptors

Both ERα and ERβ are classified as nuclear receptors and sub-classified in the steroid receptor family, sub-family 3, group A (Nucleur Receptors Nomenclature Committee, 1999). The receptors are resident in the nucleus, whether free or bound to ligand (Parker 1995; Figure 1.5, page 9). ERα was first identified and cloned from the rat uterus in 1987; the molecular weight of the protein is approximately 67 kDa (Koike, 1987), it shares 88% homology with the human ERα. An additional nine years were required to identify and clone the second receptor, ERβ, this has a molecular weight of 54.2 kDa (Mosselman *et al.* 1996). The two receptors have a similar over all homology; both contain six regions, termed A-F. Figure 1.4 illustrates the homology between the receptors; the greatest homology is within the DNA and ligand binding domains (domain C and E respectively; Kuiper *et al.* 1996; Tremblay *et al.* 1997). The ligand binding domain is the region of the receptor that hormone or ligands bind to (Tsai *et al.* 1994). The high homology in the ligand binding domain is evident from the similar binding kinetics of ligands, $17\beta E_2$ has a K_i of 0.13nM and 0.12nM for ERα and ERβ respectively (Kuiper *et al.* 1997). The high homology in the DNA binding domain allows ERα and ERβ to recognise and bind to a specific pallindromic DNA sequence, known as the E response element (ERE) in the target gene.

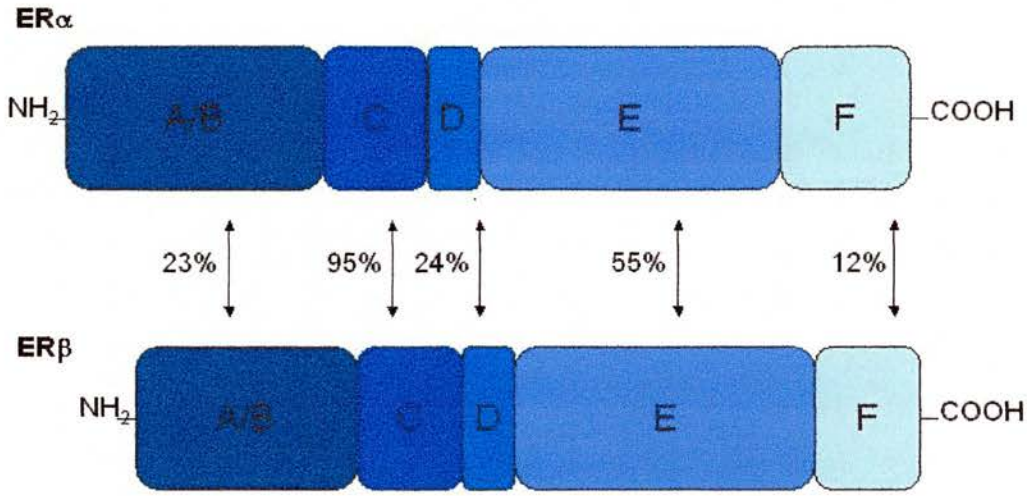


***Figure 1.4.* ERα and ERβ and the relative homology between them.**
Domain A contains the transcription activation factor AF-1, domain C is responsible for DNA binding and domain E for ligand binding and contains transcription activation factor AF-2. (Taken from Ness, 2005)

The ER is an inactive monomer bound to a chaperone such as a 'heat shock protein' (HSP). Upon ligand binding, a conformational change causes the HSP to dissociate and the receptors to dimerise, this dimerisation can be homologous or heterologous. The conformational change promotes and stabilises the interaction of ER with other co-factors. The co-factors may be activators or repressors, activators link the ER with histones or components of basal transcription, whilst repressors attenuate transcription by competing with activators, they also associate to the ER in the presence of antagonists (Nilsson *et al.* 2001).

The co-factors bind to transcription activation factor-1 (AF-1) in the regulatory domain (domain A) or AF-2 within the ligand binding domain (Ruff *et al.* 2000). AF-1 activity is ligand independent, dependant on the cellular environment and regulated by growth factors. AF-1 may undergo phosphorylation and forms a site for interaction of transcription factors, it is also involved in protein-protein interactions (Nilsson *et al.*

2001). In contrast to AF-1, AF-2 binds both agonists and antagonists to cause a conformational change, AF-2 activity is solely dependant on ligand binding (Pike *et al.* 2001). Variations in ERα and ERβ activation and responses are thought to arise from cell specific co-activators and co-repressors, low homology at the amino terminus end and different AF-1 and AF-2 expression (Hall *et al.* 2001). Human ERβ does not contain an AF-1 domain, where as mouse ERβ does, this makes comparison studies more difficult to interpret (Hall *et al.* 1999).

Crystalisation studies have revealed the importance of AF-2 in the determination of ER response to ligand binding. Within AF-2 there are a number of helix motifs which cover and line the binding cleft (Brzozowski *et al.* 1997; Pike *et al.* 1999). Upon ligand binding the position of these helixes changes, the crystalisation studies have highlighted the importance of helix 12. The conformational position of helix 12 is a major determinant of ligand activity. When ERα is bound to an agonist, such as $17\beta E_2$ helix 12 forms the binding site for co-activators (Brzozowski *et al.* 1997). In contrast, when an antagonist such as raloxifene is bound to the receptor helix 12 is displaced, co-activators are no longer able to bind to the receptor-ligand complex (Brzozowski *et al.* 1997; Reviewed by Nilsson *et al.* 2001). After dimerisation the receptor-ligand complex acts as a transcription factor on a wide range of genes. ERα and ERβ have distinct but over lapping cellular localization, within a tissue each receptor may be localized to a different cell type, thus producing varied responses. Both receptors are expressed within the cardiovascular system (CVS; Gustafsson 1999).

There is also an ERE independent pathway of transcription regulation, where E interacts with the DNA indirectly. The activated ER forms a complex with DNA-bound transcription factors through the AP-1 binding site and therefore does not require an ERE. In addition to these genomic pathways there is also a non-genomic pathway discussed in Section 1.2.1.2. (Taylor *et al.* 2000; Figure 1.5).
Recently receptor specific ligands have been developed to selectively activate (Herrington *et al.* 2003b) or antagonise the receptor of interest. Prior to these selective

ligands non-selective agonists and antagonists limited the ability to investigate the individual receptors. Mice have been genetically altered to remove either ERα (ERKO) or ERβ (BERKO; Krege *et al.* 1998). Experiments with these models are limited due to compensatory and complementary mechanisms of the remaining receptor, the possibility that the receptors are not removed entirely and species difference in expression (Couse *et al.* 1995). A double knock out (KO) mouse has also been used to study the role of ERs. These experimental results with E show that some of the cardiovascular protection including inhibition of vascular smooth muscle cell (VSMC) proliferation remain despite both ERs being removed. The results suggest that there is a third receptor, these properties are ER independent, non-genomic (Karas *et al.* 2001a). There is also evidence supporting the concept of receptor splice variants in the KO animals and that these receptors may confer some of the receptor properties (Smithson *et al.* 1998). For the reasons stated here and the limitations of the KO mice in the work carried out during this PhD we employed novel ER selective agonists and antagonists.

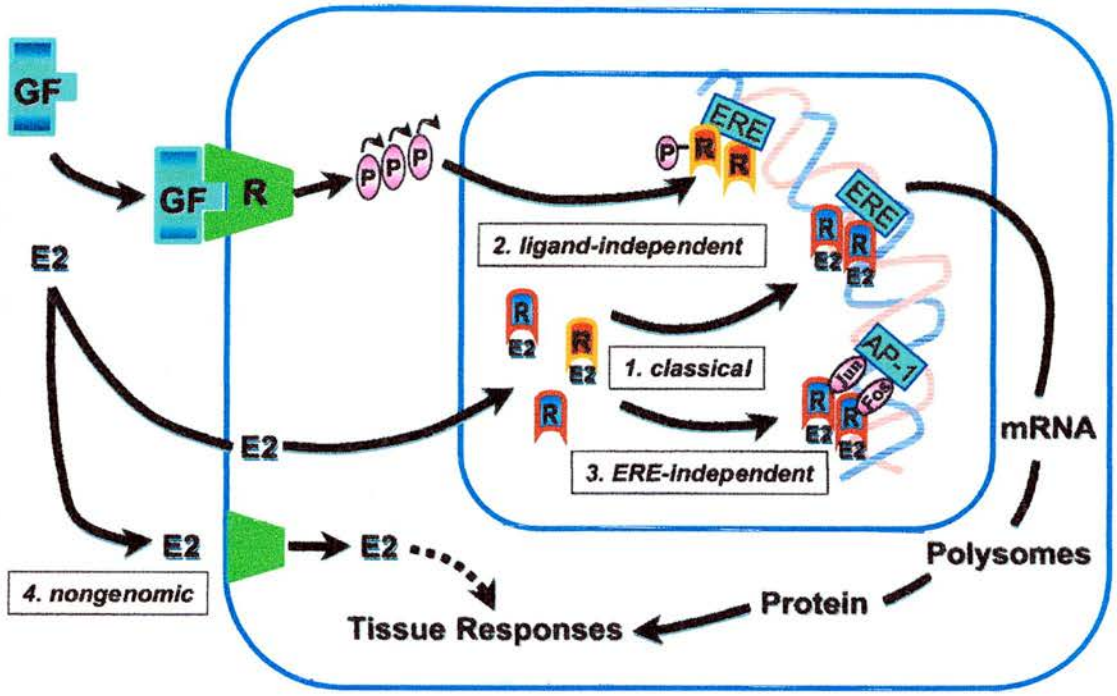


Figure 1.5. **The classical and non-classical pathway of oestrogen signalling within the cell.**

(1.) The classical activation requires E to diffuse into the cell across the cell membrane and enter the nucleus where it binds to ERs. Binding of E to ERs causes a conformational change enabling the receptor-ligand complex to dimerise and bind to the oestrogen response element (ERE) on DNA. (2) The ER can be activated and bind to the ERE by phosphorylation, independent of ligands. (3) Once the ER is activated by ligand binding it is able to act independently of the ERE through other sequences with in the DNA such as the AP-1 site. ***(4.)*** *In the non-genomic pathway E interacts with ERs on the cell membrane that activates intracellular signalling pathways. The non-genomic pathway results in a rapid response, within seconds to minutes compared to the classical genomic activation that creates a response in minutes to hours.*(Hall *et al.* 2001)

1.2.1.2. Non-genomic properties of oestrogen

The idea of a non-genomic ER is not new. The non-genomic responses of E are observed within minutes and hours of administrating E. Studies 30 years ago with E conjugated to bovine serum albumin (BSA), which is too large to penetrate the cellular membrane, demonstrated that E has cell surface receptors which are responsible for the rapid non-genomic effects of E (Pietras *et al.* 1977, 1980). Since these studies fluorescence microscopy has shown $17\beta E_2$ bound to BSA at the cell surface (Kim *et al.* 1999) and immunoblot studies have detected ERα 67 kDa epitopes at the cell membrane (Chambliss *et al.* 2000). Within the CVS immunofluorescent studies show staining on both the nuclear and cytosolic membranes of cardiomyocytes and fibroblasts indicating that these cells posses ERs in both locations (Grohe *et al.* 1997).

Numerous studies have been carried out to try to identify and characterise such a receptor (Chen *et al.* 1999; Pappas *et al.* 1995; Wehling 1997). The structure of the membrane ER has not been determined, but transfection of Chinese hamster ovary cells has demonstrated that a single RNA and cDNA is capable of synthesising both the membrane and nuclear receptors (Razandi *et al.* 1999). Debate arises because the ER gene does not encode a hydrophilic domain for anchorage into the plasma membrane. The ER may be tethered through glycophosphatidly-inositol links or localised to the caveola signalling transduction and vesicular trafficking domains in the plasma membrane (Reviewed by Losel *et al.* 2003). The membrane receptor is most likely to be G-protein linked and activate signalling pathways accordingly (Razandi *et al.* 1999). Recently striatin has been identified as a key protein essential in non-genomic E mediated response and the formation of a complex to G-protein coupled pathways (Lu *et al.* 2004). Over expression of striatin in cultured cells causes a shift in the cellular distribution of ERs towards the cell membrane (Lu *et al.* 2004). These studies suggest the ERs may be 'pulled' to the plasma membrane from the nucleus.

Orphan E related receptors have recently gained experimental interest within cancer biology, these receptors are closely linked to ERs and bind to EREs (Ariazi *et al.* 2006). At the moment this research has concentrated on these receptors in cancer and not CVD (Sun *et al.* 2006).

1.3. Oestrogen and the cardiovascular system

Clinical observation studies (Reviewed by Herrington *et al.* 2003a) and experimental models have demonstrated the beneficial properties of E within the CVS (Reviewed by Mendelsohn *et al.* 1999). These experiments have determined some of the cardiovascular parameters that E modulates, which include both genomic and non-genomic properties.

Both ERα and ERβ are expressed in cardiac myocytes and fibroblasts in the rat (Grohe *et al.* 1998), mouse (Gustafsson 1999) and human (Mendelsohn *et al.* 1999). Within rat and human vasculature both ER's are expressed in the smooth muscle cells (Mosselman *et al.* 1996; Nakamura *et al.* 2004) and endothelial cells (Simoncini *et al.* 2004b). ERα is also expressed within fibroblasts (Mahmoodzadeh *et al.* 2006).

It is well known that E protects against atherosclerosis and plaque formation within the vasculature (Mendelsohn *et al.* 1999). Within the CVS E favourably alters the ratio of plasma lipid levels by decreasing the level of low density lipoproteins (LDLs) and simultaneously increasing the high density lipoproteins (HDLs). E also acts as an antioxidant reducing oxidative damage of myocardial cells and preventing the oxidation of LDLs. E moderates endothelial function and accelerates endothelial cell growth, it also enhances vasodilation in the presence and absence of intact endothelium. E is anti-apoptotic preventing cell death and consequently provides protection against atherosclerosis and plaque formation. Through enhanced nitric oxide (NO) release E also decreases blood pressure (Reviewed by Mendelsohn *et al.* 1999). After injury and during periods of inflammation E reduces the inflammatory response through decreased

expression of vascular adhesion molecules and inhibiting VSMC proliferation. Membrane receptors mediate a large number of E responses within the CVS, these include enhanced NO release, inhibited cardiomyocyte apoptosis and hypertrophy (Kim *et al.* 2006). These properties of E within the CVS will be discussed in more detail in relation to their response in MI.

1.3.1. Endothelial function including Nitric Oxide release, cell proliferation and the role of oestrogen

Endothelial cells provide a protective barrier against clot formation and release vasodilators, such as NO, that mediate vascular tone (Mullane *et al.* 1987). Endothelial dysfunction and injury are thought to be early initiators of atherosclerosis. Impaired NO release during endothelial dysfunction contributes to disease progression. VSMC proliferation during endothelial dysfunction is an important step in lesion formation and atherosclerosis (Ross *et al.* 1973). NO released from the endothelium not only acts as a vasorelaxant, but is also involved in endothelial regeneration, reduction in platelet adhesion and inhibits leukocyte chemotaxis (Barbato *et al.* 2004). Rapid release of NO and vasodilation is the predominant non-genomic property of E within the CVS. Administration of E to ovariectomised (ovx) animals and primates *ex vivo* and *in vivo* results in rapid non-genomic vasodilation within minutes (Sudhir *et al.* 1997; Williams *et al.* 1992). Experimentally, in cultured endothelial cells this response to E is blocked by ER antagonists and ERα stimulation directly activates eNOS (Chen *et al.* 1999; Gilligan *et al.* 1994). Increased NO decreases blood pressure, platelet aggregation, leukocyte adhesion, vascular smooth muscle mitogenesis (Moncada *et al.* 1993) and inhibits VSMC proliferation (Chen *et al.* 1996; Leiberman *et al.* 1995).

Studies investigating the effect of E on the function of the endothelium in humans have employed forearm blood flow analysis (Higashi *et al.* 2001; Lima *et al.* 2005). These studies have demonstrated that treatment with E increases forearm blood flow in postmenopausal women, thus E stimulates vasodilator release from these cells *in vivo* as

it does *in vitro* (Gerhard et al. 1998). Menopause and a decrease in E levels is accordingly associated with endothelial dysfunction; an early predictor of coronary artery disease (Mercuro *et al.* 1999a).

1.3.2. Serum lipids and oestrogen

High serum lipid levels and cholesterol are risk factors for developing atherosclerotic plaques within coronary arteries. Pre-menopausal women have reduced atherosclerosis compared to their age matched male counter parts (Mendelsohn *et al.* 1999). This sex related difference in plaque formation is accounted for by E (The writing group for the PEPI trial 1995). Through regulation of hepatic apoprotein genes E decreases plasma levels of LDLs, cholesterol and serum Lp(a) lipoproteins, whilst simultaneously increasing the circulating levels of beneficial HDLs (Kushawaha 1992; Reviewed by Mendelsohn *et al.* 1999). The ability of E to alter the ratio of plasma lipids is thought to account for around a third of its cardiovascular benefits (Bush *et al.* 1987; Mendelsohn *et al.* 1994)

1.3.3. Oxidation and oestrogen

Oxidation of LDLs enhances vascular damage through chemotactic and cytotoxic properties and the formation of foam cells. Oxidative damage plays a significant role in a range of human CVDs, including MI, ischaemia-reperfusion and atherosclerosis (Darley-Usmar *et al.* 1997). Systemically E acts as an anti-oxidant and suppresses free radical induced peroxidation through its phenoxyl radical (Sugioka *et al.* 1987). E also regulates the expression of genes that regulate local production and degradation of superoxide, including superoxide dismutase (SOD) and glutathione (Strehlow *et al.* 2003). E also improves the ratio of NO to nitric dioxide (NO_2) through down regulation of NADPH oxidase in human endothelial cells (Wagner *et al.* 2001).

1.4. Myocardial Infarction

MI is caused by a lack of oxygen and nutrients to part of the coronary muscle. This is commonly caused by the rupture of an atherosclerotic plaque which exposes a thrombotic surface within a coronary artery (Corrado *et al.* 1994). It is now recognised that the pathology of MI and sudden death is different in pre-menopausal women, where it is due to plaque erosion, compared to the distinct process of plaque rupture in males and post-menopausal women (Burke *et al.* 1998). The recent Women's Ischaemia Syndrome Evaluation (WISE) study identified the atypical symptomatic nature of presentation of CHD in women (Quyyumi 2006). Twenty percent of the women studied had evidence of myocardial ischaemia with chest pain, but had normal coronary arteries on angiography. The defect in these women seemed to lie in the coronary microvasculature.

1.5. Myocardial remodelling post-myocardial infarction

MI results in cell death leading to muscle loss and consequently decreased function of the heart. If severe the loss of myocardial function causes arrhythmias and immediate death. In less severe cases of MI survival is more likely and there is a healing process to replace the damaged and necrotic tissue.

The acute remodelling process lasts for approximately four days and is a combination of overlapping and complementing mechanisms, including an inflammatory response, new tissue formation and tissue remodelling including collagen deposition and then angiogenesis (Frangogiannis *et al.* 2002; Kawakami *et al.* 2004). Scar tissue formation to replace necrotic myocardial tissue is one of the main remodelling processes following MI. Acute remodelling is involved in cardiac failure, arrhythmias and cardiac rupture. These conditions all develop through the degradation of matrix proteins and collagen from within the extracellular matrix (ECM; Takano *et al.* 2003).

1.5.1. The extracellular matrix

The ECM is a dynamic tissue comparable between all mammalian species and is fundamental to most tissues and organs, although the composition of it varies between organs (Williams 1989). The ECM provides physical support and structure to tissues. The ECM also determines the three dimensional organisation of the cells it surrounds, and hence the physical properties of tissues. Interactions between cells and the ECM influence cell signalling which can directly manipulate cell behaviour and function within remodelling thus effecting differentiation, proliferation, growth and survival of cells (Lukashev *et al.* 1998; Ogawa *et al.* 2000). In most tissues, including the myocardium the ECM consists of fibrillar collagen, elastin, microfibrillar proteins, proteoglycans and adhesive proteins such as laminin (Fedak *et al.* 2005). The myocardial ECM consists predominantly of fibrillar collagen, of this approximately 85% is type I. One of the major properties of collagen type I is high tensile strength which helps to prevent over stretching and deformation of the ECM. Collagen type III accounts for approximately a further 11% of the total collagen, this has thin fibres providing the ECM with its resilience (Assoian *et al.* 1997; Buck *et al.* 1993; Hornberger *et al.* 2000; Ross *et al.* 2001; Simpson *et al.* 1994).

The ECM is able to alter both its composition and organisation to have profound effects on the surrounding tissue. Diseases resulting from gene deletion or mutation within the ECM have demonstrated the major role the ECM has on organs within the body and the diverse range of organs that are effected by it (Burlew *et al.* 2000). Major CVDs including left ventricular hypertrophy from volume overload, dilated cardiomyopathy, MI and hypertension are all affected by altered ECM composition. Both human and animal studies have shown that during remodelling post-MI there are changes in left ventricle (LV) collagen content (Cleutjens *et al.* 1995b; Sackner-Bernstein 2000). The amount, type, stability and organisation of the fibrillar collagen within the ECM are all likely to play a key role in these diseases (Ju *et al.* 1996). An excess of collagen is typically laid down in the infarcted myocardium during remodelling post MI. The

increased collagen content forms a stable scar to provide protection and prevent the weakened area from cardiac rupture (Sun *et al.* 2000b).

Despite the main function of the myocardium and its ECM being to work as a muscular pump cardiomyocytes only account for around 33% of the total cells, although they occupy about 66% of the myocardial volume (Zak *et al.* 1973). The most abundant cell within the myocardium are fibroblasts (Robinson *et al.* 1983), their primary role is the synthesis and regulation of components of the ECM (Eghbali *et al.* 1988; Zak *et al.* 1973), the abundance of these cells suggests that there is a continuous need for new components of the ECM.

1.5.1.1. Extracellular matrix degradation

Physiologically the myocardial ECM is constantly turning over at a rate of approximately 0.6% per day. Continuous turnover of the ECM is essential for the physiological active metabolism in the myocardium and blood vessels (Weber 1989). Although not rapid this continuous turnover suggests there is a constant requirement for low levels of proteolytic enzymes. Degradation of the ECM in the myocardium is predominantly through serine and metal ion proteinases, such as matrix metalloproteinases (MMPs; reviewed in Fedak *et al.* 2005; Mott *et al.* 2004).

1.5.2 Plasmin

Serine proteinases are released from neutrophils and macrophages during an inflammatory response, inactive plasminogen is also present in the ECM. Active plasmin is a potent serine protease that hydrolyses bonds between arginine and histamine. Plasmin plays a key role in remodelling post MI (Knoepfler *et al.* 1995; Rohde *et al.* 1999) and activates growth factors released from the ECM including vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF; Ferrara *et al.* 1993).

There are two plasminogen activators (PA) that hydrolyse plasminogen into plasmin. The two PAs, urokinase-PA (u-PA) and tissue-PA (t-PA) are associated with different physiological roles within the body (reviewed by, Dano *et al.* 1985; Mignatti; Saksela *et al.* 1988). Active plasmin is capable of degrading the majority of ECM components. The development of mice with genes of the plasminogen system KO has helped ascertain the role and importance of various components of the plasminogen system during the process of cardiac remodelling (Creemers *et al.* 2000; Heymans *et al.* 1999; Knoepfler *et al.* 1995). Mice with the plasminogen gene disrupted have delayed infarct healing after MI (Creemers *et al.* 2000). u-PA KO mouse have impaired healing and are predisposed to heart failure after MI (Heymans *et al.* 1999). Administration of PA inhibitor (PAI-1) through adenoviral gene transfer prevented cardiac rupture after MI (Heymans *et al.* 1999). These studies have demonstrated that the plasminogen system is a prerequisite for the disruption of collagen and essential for functional recovery after MI. Collagen disruption is vital for cell infiltration enabling wound healing but is also the initial stage in rupture. These studies reported a decrease in MMP-2 and MMP-9 activity and significantly reduced LV wall thinning in KO mice compared to wild types, thus illustrating that plasmin activates these MMPs within the myocardium (Creemers *et al.* 2000; Matrisian 1992).

1.5.3. Matrix Metalloproteinases

The MMPs are a family of over 20 zinc-dependent proteases. Most MMPs are secreted, but recent research (Miyamori *et al.* 2000) has identified a sub-group with an additional domain enabling anchorage in the cell membrane. There are five sub-groups of MMPs, and MMPs are numbered in the order of their identification. The five sub-groups comprise of the collagenases (MMP-1, -8 and -13), capable of cleaving intact fibrillar collagen, mice naturally express MMP-13 as the major collagenase compared to humans who express MMP-1. The gelatinases (MMP-2 and -9) cleave fibrillar collagens further and basement membrane type IV. The stromelysins (MMP-3, -10 and -11) have a broad substrate specificity including fibronectin, laminin and proteoglycans, the matrilysins (MMP-7) also have broad specificity including elastin, enactin and osteopontin. The

membrane bound MMPs (MT-MMPs) have collagenolytic activity (Ohuchi *et al.* 1997) and activate other pro-MMPs (Butler *et al.* 1997). Table 1.2 highlights some of the main MMPs after MI.

Number	Latent Size (kDa)	Active Size (kDa)	Substrate Specificity	Produced from post-MI
MMP-1	55	45	Fibrillar collagens, casein, gelatin, proteoglycan	Fibroblasts
MMP-2	72	66	Gelatin, denatured collagen, collagens IV, V, VIII	Macrophages, myofibroblasts, myocytes
MMP-3	57	45	Procollagens, gelatin, laminin, proteoglycan, collagen X, XI, collagenase,	Myocytes
MMP-7	28	19	As MMP-3 and elastin	Undefined
MMP-8	75	58	Proteoglycan, fibrillar collagens, gelatin	Neutrophils
MMP-9	92	86	Gelatin, collagens IV, V, VIII, X, denatured collagens	Neutrophils, macrophages, myocytes
MMP-13	66	48	Fibrillar collagens, aggrecan, gelatin	Undefined
MMP-14	-	-	Collagen I, gelatin	Fibroblasts, VSMC and myocytes

Table 1.2. **MMPs identified post-MI** (Modified from Vanhoutte *et al.* 2005).

1.5.3.1 The structure of matrix metalloproteinases

All the MMPs share common domains. The catalytic protease domain is maintained throughout the MMP family, this domain contains the catalytic Zn^{2+} atom at the centre that is essential for cleaving collagen. The gelatinases have an additional fibronectin-type II domain, which enables interactions with gelatin and collagen (Allan *et al.* 1995). The signal pro-domain is responsible for maintaining the latency of MMPs and is conserved throughout all MMPs. The pro-domain contains a 'cysteine (Cys) switch', the Cys ligates the catalytic Zn^{2+} which maintains the protease in a latent state until cleaved (Becker *et al.* 1995; Van Wart *et al.* 1990). The haemopexin domain, consists of four anti-parallel β-strands and an α-helix to facilitate cleavage of the triple helical interstitial collagens (Bode 1995). Differences in the topology of the active site cleft and differing numbers of structural domain repeats produces MMP substrate specificity and differences in tissue inhibitor of MMPs (TIMP) activity (Nagase *et al.* 1999). The similarities between the sub-groups is illustrated in figure 1.6.

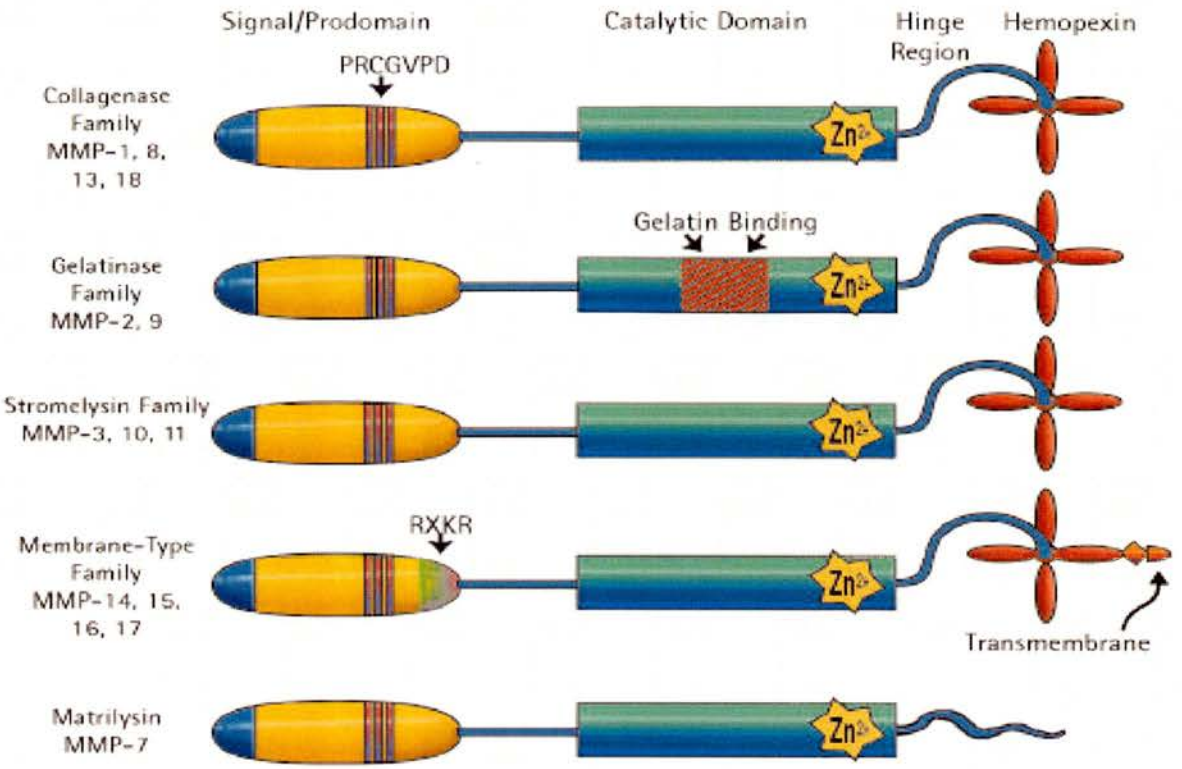


Figure 1.6. **The structure of MMPs.**
The subunits of the MMP sub-groups are highlighted. It is clear to see the similarities and differences between them, this provides the sub-groups with a degree of substrate specificity. Taken from merckbioscience.

1.5.3.2. Regulation of matrix metalloproteinases

Collectively the MMPs are capable of degrading the entire ECM and are thus vital in physiological and pathological tissue remodelling. Pathologically MMPs are involved in a wide range of conditions including cardiac remodelling (Curran *et al.* 1999; Lichtinghagen *et al.* 2001; Pap *et al.* 2000; Spinale 2002).

Stringent MMP activity regulation at three levels prevents excessive detrimental and inappropriate activity of MMPs (Figure 1.6; Cleutjens *et al.* 2002). The first regulatory step is gene expression and transcription. Cytokines such as tumour necrosis factor (TNF)-α, interleukin (IL)-1 and IL-6 stimulate MMP expression (Alexander *et al.* 1998;

Nagase *et al.* 1999). Physical stress, cell-matrix and cell-cell interactions can also induce MMP activity (Biswas *et al.* 1995), whilst factors such as glucocorticoids suppress MMP expression (Fini 1998).

Cleavage of latent-MMP and disruption of the Cys-switch is the second level of regulation. Step-by-step cleavage is the predominant mechanism of activation. Cleavage of the pro-domain causes a conformational change exposing the activation site. A second cleavage commonly by another MMP, such as MMP-3 then fully activates the MMP (Mann *et al.* 1998). MMPs are also activated at the cell surface by MT-MMPs, which cleave and activate other MMPs. MMP-2 is activated at the cell surface through a trimolecular complex consisting of pro-MMP-2, TIMP-2 and MT1-MMP (Butler *et al.* 1998). The third mechanism is the intracellular activation of MMPs, although few MMPs are activated this way and the full mechanism is poorly understood (Pei *et al.* 1995; Sato *et al.* 1996).

Finally active MMPs are inhibited by TIMPs. The TIMPs are the only inhibitory mechanism of MMPs once activated. TIMPs are expressed by a large variety of cells and found in most tissues and body fluids (Reviewed by Fedak *et al.* 2005).

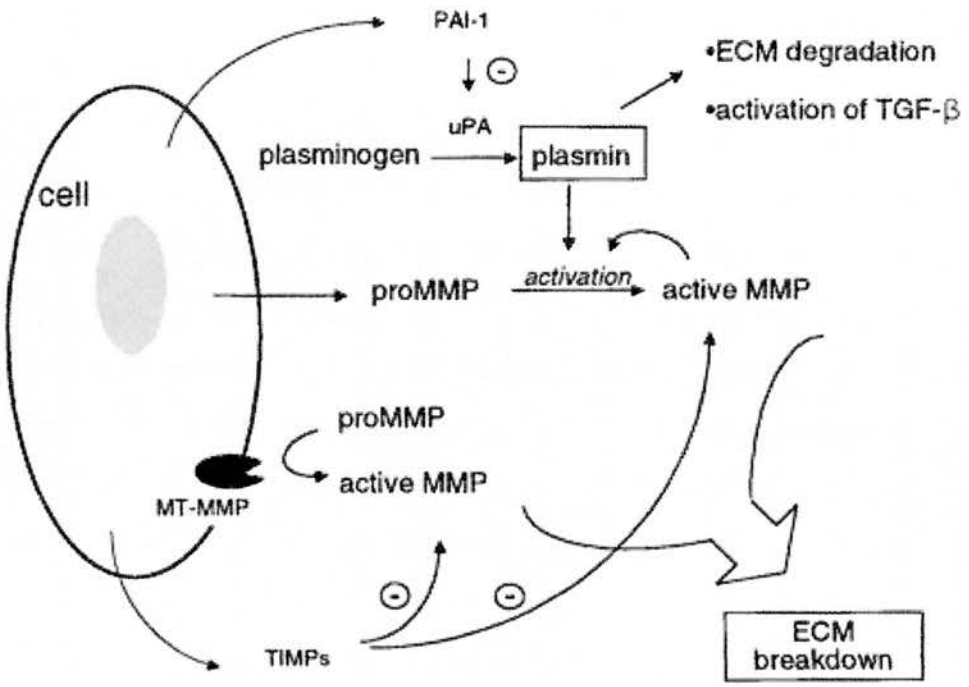


Figure 1.7. **Pathways of MMP release and regulation.**
The regulation of MMP activity involves three pathways. The initial regulation is within the cell and the rate of gene transcription Once secreted proMMPs are cleaved by plasmin (and other proteases such as trypsin and elastase). Membrane bound MMPs may also activate MMPs at the cell surface. TIMPs and PAI-1 inhibit the activation of MMPs, either directly or through the inhibition of plasminogen cleavage. The balance of MMP activation and inhibition regulates the degradation of the ECM (Creemers et al. 2001).

1.5.3.3. Tissue inhibitors of matrix metalloproteinases

TIMPs inhibit MMPs by forming non-covalent bonds at the active site of MMPs. The binding of TIMPs and MMPs has a stoichiometric ratio of 1:1; therefore the ratio of MMPs to TIMPs is important in determining the amount of MMP activity (Goldberg *et al.* 1989).

The TIMPs are a family of four proteins, which share a 40 - 52% sequence homology. A conserved tertiary structure assists in largely indiscriminate binding to MMPs (Sato *et al.* 1996; Willenbrock *et al.* 1994). TIMP-1 and -3 are readily inducible, TIMP-2 is constitutively expressed whilst TIMP-4 has higher expression in the myocardium (Fedak *et al.* 2005). The TIMPs consist of two structurally and functionally distinct domains,

the N- and C- terminal domain. The N terminal domain is the main inhibitory unit that interacts with the Zn^{2+} binding site of MMPs. The C terminal domain also interacts with MMPs, these interactions are thought to ameliorate TIMP inhibition of MMP activity (Willenbrock *et al.* 1994; Williamson *et al.* 1990). Studies using specific peptides and antibodies to block regions within TIMPs have helped identify the function of distinct areas (Bodden *et al.* 1994) while x-ray crystallography studies have aided understanding of the structural changes that occur upon complexes forming between MMPs and TIMPs (Gomis-Ruth *et al.* 1997). Similar studies have also demonstrated the ability of TIMP-2 to bind to MT1-MMP (Fernandez-Catalan *et al.* 1998). The binding of TIMP-2 with MT1-MMP displays a unique property of TIMP-2, enabling it to cleave and activate pro-MMP-2 (Kolkenbrock *et al.* 1991).

1.5.3.4. Matrix metalloproteinases in extracellular matrix degradation.

The MMPs are the predominant regulatory mechanism controlling the degradation and turnover of the myocardial ECM following an episode of MI and accordingly have gained a wealth of experimental interest (Reviewed by Fedak *et al.* 2005; Vanhoutte *et al.* 2005). In addition to degrading the ECM, MMPs also stimulate cytokine release to promote fibroblast migration and proliferation. This stimulation is necessary for synthesis and deposition of new ECM and scar tissue (Lindsey *et al.* 2001; Petrov *et al.* 2002). Exacerbated activation of MMPs and therefore ECM degradation post-MI may also have detrimental consequences resulting in cardiac rupture, LV dilation and dysfunction (Spinale 2002).

1.5.3.5. Matrix Metalloproteinases post-myocardial infarction

Myocardial MMP activity is increased within hours of MI and is correlated to an initial decrease in collagen content in the myocardium within 48hrs (Herzog 1998; Villarreal *et al.* 2003). In this thesis I looked at the activity of MMPs at four days after MI when myocardial remodelling is thought to be at an optimal rate. The activity of MMPs is described as being in the acute remodelling period post-MI. Although this is not in the

immediate period post-MI, it was the acute remodelling period in comparison to the remodelling that is observed weeks and months after the initial infarct.

MMP-2 and MMP-9 have gained the most experimental interest post-MI. Myocardial activity of MMP-2 and MMP-9 is increased during the first seven days post MI (Lindsey *et al.* 2001; Lindsey *et al.* 2002; Nagaoka *et al.* 2000). Activated macrophages, fibroblasts and myocytes are all likely sources of MMP-2 during the remodelling period after MI (Table 1.2; Heymans *et al.* 1999; Romanic *et al.* 2001). Increased MMP-9 protein synthesis is associated with active neutrophils (Nagaoka *et al.* 2000). Lindsey et al have demonstrated that active MMP-9 is co-localized with infiltrating neutrophils post-MI (Lindsey *et al.* 2001). MMP activity is still raised during the granulation period and chronic remodelling (Pfeffer *et al.* 1990) although gelatinase activity begins to decline up until day 14 (Peterson *et al.* 2000). Other MMPs that have increased activity after MI and in the failing heart include MMP-3 and MMP-1, which has increased expression 2-7 days after MI in the rat model (Cleutjens *et al.* 1995b; Spinale *et al.* 2000; Thomas *et al.* 1998). The differences in MMP activation profiles after MI suggest that they have different functions within the ECM break down and remodelling process, including allowing inflammatory cell infiltration into the infarct region.

The difference in the cellular origin of MMPs, such as MMP-8 and MMP-9 from neutrophils (Lindsey *et al.* 2001) and the MMP-2 from cells constitutively expressed in myocardium is likely to play a significant role in the temporal expression of the proteinases in remodelling (Tao *et al.* 2004). Therefore the substrates of specific MMPs will be degraded in a time ordered manor. Disrupting the order of stimulated MMP expression and activity is likely to have detrimental effects on remodelling.

1.5.3.6. Oestrogen and Matrix Metalloproteinases

The activity of MMPs and TIMPs are regulated by E in the reproductive tract and uterus (Helvering *et al.* 2005; Marbaix *et al.* 1992) as well as non-reproductive tissues including tumour cells and the CVS (Potier *et al.* 2001; Wingrove *et al.* 1998). Experimentally treatment with E increases MMP-2 activity and attenuates age related LV remodelling and ECM changes that result in collagen accumulation which is more pronounced in females (Smith *et al.* 2000; Xu *et al.* 2003). The role of E in acute remodelling post-MI is not known, the possibility that E regulates MMP expression and activity, either directly or in-directly post-MI and consequently results in the detrimental remodelling that leads to cardiac rupture has not been investigated to date.

1.6. The Inflammatory response

The inflammatory response is imperative for infarct healing and scar formation (Entman *et al.* 1994). The duration of coronary artery occlusion is crucial for inducing an inflammatory response; rapid reperfusion that prevents cellular injury is not associated with an inflammatory response. Continued occlusion of an artery induces infarction and concomitantly instigates an acute inflammatory response, involving a humoral response and cell infiltration. The inflammatory response differs if reperfusion occurs following the ischaemic period. In the presence of reperfusion the inflammatory response is accelerated and intensified (reviewed by Frangogiannis *et al.* 2002; Hansen 1995).

The importance of the inflammatory response in infarct healing after MI was first demonstrated in canines (Libby *et al.* 1973). The discovery that reperfusion enhances the inflammatory response and improves infarct healing (Jugdutt 1997) is supported by studies in humans with non-steroidal anti-inflammatory drugs. These studies resulted in attenuated wound healing and increased cardiac rupture post-MI (Stern MD, 1988; Solodky A, 2001). Similarly, studies with corticosteroid suppressed the inflammatory response and attenuated the healing process including scar formation (Kloner *et al.* 1978).

Myocardial cell necrosis results in the release of the mitochondria rich fragments which initiates the activation of the complement cascade (Rossen *et al.* 1994). The influx of inflammatory cells is stimulated through C5a within hours of MI. There have been numerous studies on the inhibition and depletion of the complement cascade, all of which support the notion that activation of the cascade enhances infarct healing and has a significant role in the recruitment of monocytes and neutrophils to the ischaemic region of the myocardium (Reviewed in Frangogiannis *et al.* 2002).

1.6.1. Neutrophils

The primary function of neutrophils is to destroy and remove foreign micro-organisms and inflammatory debris. Granules within the cytoplasm are required for the storage of bacteriocidal and cytotoxic species vital for the non-specific immune response (Wysocka *et al.* 2001).

The neutrophil cell membrane contains adhesive proteins, membrane channels and receptors for ligands, cytokines and chemotatic factors, as well as ectoenzymes and ion pumps (Murphy 1994). These features regulate neutrophil activation and ensure accumulation at the site of injury (Reviewed by van Es *et al.* 1999). Neutrophil activation stimulates the granules within them to fuse with phagocytic vesicles and release their contents. The granules are involved in a number of neutrophils properties during inflammation, including complement activation, leukocyte adhesion and collagen removal (Wright *et al.* 1977). Granule contents may be bacteriocidal such as myeloperoxidase (MPO); MPO catalyses the production of hypochlorite (OCl^-) from chloride and hydrogen peroxide (Paul *et al.* 1970). Neutrophil granules also contain MMP-9 and MMP-8 (Knauper *et al.* 1993). Neutrophils infiltrate the ischaemic area up to 4 days post-MI (Reimer *et al.* 1989), the proteases released in this time assist in the removal of necrotic tissue and are essential for healing and scar formation (Jordan *et al.* 1999).

1.6.1.1. Chemoattractants

Chemokines are leukocyte specific chemoattractants that allow rapid and selective accumulation of leukocytes at areas of injury and inflammation. IL-8 is thought to play a fundamental role in the accumulation of neutrophils and TNF-α plays a central role in the inflammatory cascade (Stancovski *et al.* 1997). The complement cascade and reactive oxygen species (ROS) induce release of cytokines and chemoattractants at the site of injury (Shingu *et al.* 1984).

The process of chemotatic factors binding to their respective receptors on neutrophils activates the neutrophil. Upon activation neutrophils undergo polarisation that assists their directional movement to the site of inflammation and release their granule contents (Reviewed by van Es *et al.* 1999). Once activated neutrophils release autocoids that act locally such as thromboxane A_2 and LTB_4 to promote vasoconstriction and platelet activation, they also release more cytokines such as IL-8 that acts as a positive feedback attracting more neutrophils (Ivey *et al.* 1995; Kukielka *et al.* 1995a; Sekido *et al.* 1993).

1.6.1.2. Neutrophil adhesion

Adhesion molecules, including selectins, integrins and the immunoglobulin super family mediate neutrophil adhesion to and passage through endothelial cells to ultimately achieve neutrophil accumulation at the inflamed site, as illustrated in Figure 1.7 (Frangogiannis *et al.* 2002).

The initial step of the adhesion cascade involves rapid interactions and weak bonds between selectins and their ligands to bring neutrophils into close proximity with endothelial cells (Figure 1.7; Alon *et al.* 1995; Springer 1994). The weak bonds increase neutrophil rolling at the site of inflammation, which stimulates further chemoattractant interactions (Figure 1.7). The resulting interactions between β_2 integrins and intercellular adhesion molecule (ICAM)-1 are stronger than the selectins, and consequently neutrophil adherence to the epithelium is also stronger (Lawrence *et al.* 1991). ICAM-1 expressed at the cell junction also plays an important role in the

migration of neutrophils through the cell junction (Furie *et al.* 1991; Furie *et al.* 1992; Smith *et al.* 1992). A third adhesion molecule, PECAM-1 forms interactions with integrins on neutrophils and influences neutrophil movement through the endothelial membrane and perivascular basement membrane. Neutrophils then pass into the subendothelial matrix and finally to the site of inflammation (Dangerfield *et al.* 2002; Duncan *et al.* 1999; Liao *et al.* 1995; Muller *et al.* 1993; Thompson *et al.* 2001).

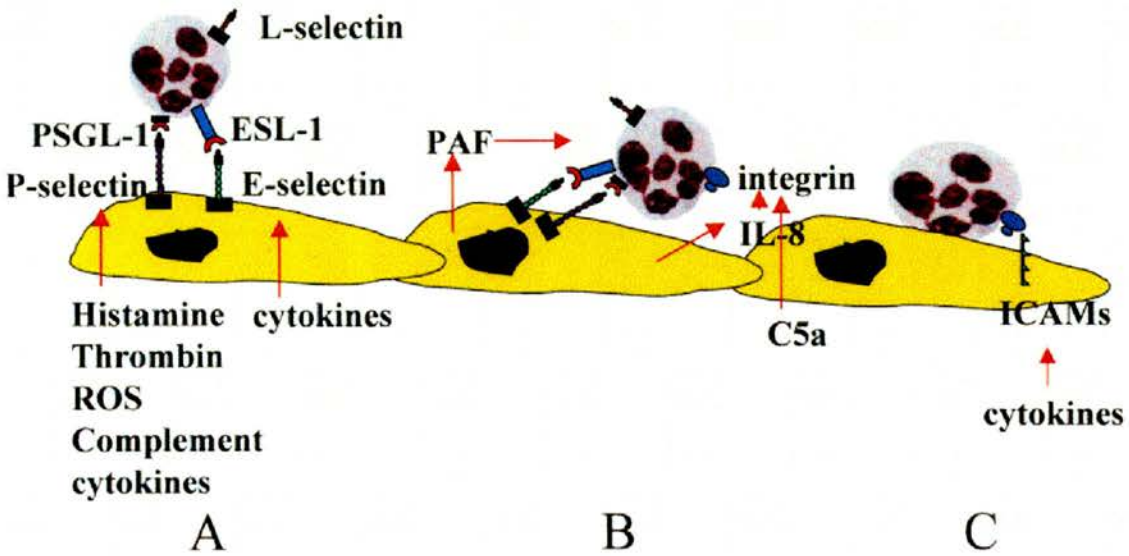


Figure 1.8. **The highly co-ordinated step process for neutrophil adhesion and transmigration through the endothelial cells to the site of injury.**
Neutrophils are activated due to the release of chemotatic mediators released by the endothelial cells. (A) The initial interaction between neutrophils and endothelial cells is a weak interaction mediated by the selectins. (B) This interaction enhances neutrophil rolling and the 'detection' of activating factors, such as IL-8 and C5a. (C) Stronger bonds are formed through integrins and ICAM-1. Once the neutrophil is firmly adhered to the endothelium it can transmigrate through the endothelium into the tissue and to the injured and inflamed site. It is here that the neutrophil will phagocytose debris from necrotic cells. (Taken from Frangogiannis et al. 2002).

Neutrophil infiltration is a prerequisite for defence against bacterial disease yet inappropriate and excessive activation plays a major contributory factor in the pathology of inflammatory diseases, such as ischaemia-reperfusion injury (Reviewed by van Eeden *et al.* 1999). The high concentration of inflammatory mediators that attract neutrophils to

the endothelium can result in endothelial cell damage, microvascular permeability and haemorrhage if left unabated. If neutrophil recruitment and cell injury is so great, the aggregating neutrophils can block small vessels and cause local ischaemia (Engler *et al.* 1983). Finally, the migration of neutrophils out of the vasculature can result in tissue damage and organ dysfunction, known as no-reflow reperfusion injury (Reimer *et al.* 1989). Despite these detrimental effects of the inflammatory response following MI, the wound healing that results from an inflammatory response is essential (reviewed in Frangogiannis *et al.* 2002).

1.6.2. Macrophages in ischaemia

Macrophages are large monocyte derived immune cells. C5a of the complement cascade is predominately responsible for the initial monocyte infiltration within hours of reperfusion, after which TGF-β1 and monocyte chemoattractant protein (MCP-1) feature (Birdsall *et al.* 1997). Differentiation into macrophages occurs after monocyte recruitment to the infarct. Differentiation is likely to be through the stimulation of Macrophage Colony-Stimulating Factor (M-CSF; Reviewed in Frangogiannis *et al.* 2002).

Macrophages play a key role in all aspects of wound healing, their prevailing function is to remove necrotic and foreign matter through phagocytosis. Macrophages release fibrogenic and angiogenic growth factors that regulate the formation of granulation tissue and angiogenesis. These factors are essential for developing a stable scar and maintaining myocardial integrity, these are discussed further in Section 1.6.4. Cytokines, such as TNF-α and fibroblast growth factor (FGF-1) released upon macrophage stimulation prevent apoptosis and thus provide myocyte protection (Trial *et al.* 2004). Monocyte secretion of MMPs and TIMPs are likely to regulate ECM degradation and scar formation, although this is only known out with the heart (Arras *et al.* 1998).

Experimental models using anti-macrophage serum to diminish macrophages result in impaired healing, decreased matrix production and fibrosis (Cohen *et al.* 1987;

Leibovich *et al.* 1975). The infiltration of macrophages, just like the neutrophils also has detrimental effects, activated macrophages release TNF-α, ROS and NO which all decrease the contractility of the heart (Simms *et al.* 1999). The role of macrophages may be beneficial in the early phases of remodelling and detrimental in the later stages of scar formation and LV remodelling.

1.6.3. Oestrogen and the inflammatory response

Experimentally E is able to inhibit inflammatory cell infiltration and activation at sites of injury, such as in atherosclerosis and after *in vivo* ischaemia-reperfusion (Booth *et al.* 2003; Delyani *et al.* 1996; Squadrito *et al.* 1997). This E mediated decrease in neutrophil infiltration and adhesion is through direct inhibition of chemotaxis and L-selectin expression. Isolated neutrophils express both ERα and ERβ (Stygar *et al.* 2006) and E directly regulates neutrophil function (Ito *et al.* 1995; Klebanoff *et al.* 1997). In cultured endothelial cells expression of neuronal NOS (nNOS) in neutrophils is up regulated by E through direct modification of the L-arginine pathway and NO synthesis (Hishikawa *et al.* 1995; Ruehlmann *et al.* 1997). The NO produced by neutrophils prevents adhesion to the endothelium, the resulting transmigration and phagocytosis leading to attenuated cell necrosis and apoptosis in the myocardium.

1.6.4. Angiogenesis

MI is associated with an early and pronounced increase in angiogenic factors including VEGF, IL-8 and bFGF, this results in an imbalance of angiogenic and angiostatic factors which favour angiogenesis (Carmeliet 2000; Kukielka *et al.* 1995b; Lee *et al.* 2000; Li *et al.* 1996). Angiogenesis is crucial for supplying the infarcted myocardium with oxygen and nutrients, experimentally this reaches a maximum at day 7 post-MI in the rat (Sherry 2000) and mouse (Small *et al.* 2005). Angiogenesis depends on interactions between the ECM and endothelial cells. Degradation of the ECM is essential for angiogenesis, it allows new vascular endothelial cells to pass through the basement membrane and

migrate through the ECM prior to dividing and organizing into new vessels (Moscatelli *et al.* 1988).

1.6.5. Fibroblasts and collagen formation

Physiologically fibroblasts regulate the ECM through the synthesis of it's components; including pro-collagens and the cross linking enzyme lysyl oxidase (Reynaud *et al.* 1999). Fibroblasts also synthesis and release MMPs (Heymans *et al.* 1999; Romanic *et al.* 2001), capable of degrading the ECM, therefore they maintain the integrity of the ECM. Fibroblasts reside in the myocardium, upon stimulation, such as MI they differentiate into myofibroblasts (Brown *et al.* 2005). Myofibroblasts are a unique cell type that express contractile proteins but are yet more motile than smooth muscle (Baudino *et al.* 2006). Fibroblasts express membrane CD19 and discondin domain receptor 2, it is possible to utilise this expression to measure fibroblast infiltration and accumulation at the site of injury, such as the infarct (Squires *et al.* 2005). ERα also is known be expressed in fibroblasts and E increases their proliferation (Lee *et al.* 1998).

Fibroblasts infiltrate the infarcted region from three days after MI and are abundant by day seven, which coincides with the peak remodelling phase (Sun *et al.* 2000a). At this time the proliferation and differentiation of fibroblasts is greatly increased at the infarct border (Squires *et al.* 2005). Post-MI, fibroblasts synthesize ECM components, namely collagen, fibronectin and laminin to replace the necrotic myocytes (Weber 1989). The fibroblasts regulate the synthesis of matrix components and co-ordinate the response to MI and pressure changes within the heart (Kanekar *et al.* 1998). Some of the infiltrating fibroblasts differentiate into myofibroblasts which allows the cells to function as a contractile unit in the myocardium (Frangogiannis *et al.* 2000). A collagen rich scar is the ultimate outcome of remodelling post-MI. The ultimate wound healing post-MI is a collagen rich scar to replace the necrotic myocytes and maintain the integrity of the LV. Experimentally a collagen rich scar is clearly visible from day seven (Cleutjens *et al.* 1995a).

The cumulative amount of collagen within the scar post-MI is not an accurate measure of the scar stability. The collagens synthesised within the infarct area vary in their functional properties, collagen type I is the predominant collagen, this had high tensile strength compared to type III, which is synthesised by 'young' fibroblasts (Weber 1989). Along with the type of collagen synthesised the cross linking is just as vital. Cross-linking of collagens is fundamental to their tensile strength and support (Balestrini *et al.* 2006; Brasselet *et al.* 2005). Analysing the collagen cross-links is therefore as informative as the amount of collagen.

1.7. Fibrolytics and Reperfusion injury

Reperfusion of the myocardium results in an extensive remodelling period which leads to a number of complications including cardiac rupture most commonly within days of MI and heart failure within years of the initial infarct (O'Rourke 1973; Solomon *et al.* 2002). The recent introduction of thrombolytic drugs such as streptokinase, administrated as soon as possible after MI have enhanced survival post-MI when prophylactic measures such as coronary artery bypass grafts, and angioplasty have failed (Blankesteijn *et al.* 2001).

Animal studies and patient profiles demonstrate advantageous results of early reperfusion, including decreased infarct size and mortality (Reviewed in Bolli 1991). Conversely, the restoration of blood flow and therefore oxygen to infarcted tissue causes detrimental functional and morphological changes in coronary arteries and myocardial tissue that result in cellular injury not previously caused from ischaemia (McCord 1985). The reperfusion injury paradox limits the extent of functional recovery after a period of ischaemia (Jeroudi *et al.* 1994). Reperfusion injury depends on the duration of ischaemia, the collateral flow during reperfusion, neutrophil influx and free radical generation in the effected region. The generation of oxygen derived free radicals, neutrophil initiated damage, loss of antioxidant enzymes, calcium overload, loss of physiological ATP concentrations, vascular endothelial and myocyte oedema and

haemorrhage are all thought to contribute to reperfusion injury. Of all these mechanisms, neutrophils and oxidative damage have generated the most interest (Jeroudi *et al.* 1994).

Experimentally 45 minutes of myocardial ischaemia is sufficient to cause irreversible damage and stimulate an inflammatory response within minutes of reperfusion which results in reperfusion injury (McCord 1985; Park *et al.* 1999). Injury in this model is most likely due to increased neutrophils in the sub-endothelial space, that can directly interact with endothelial cells (Jordan *et al.* 1999). Upon reperfusion, neutrophils invade the myocardium, release superoxide anions, hydrogen peroxide and hydroxyl free radicals in an oxidative burst (Bolli 1991; Jeroudi *et al.* 1994; Jordan *et al.* 1999). The predominant free radicals released from neutrophils during oxidative stress are superoxide anions (Tauber *et al.* 1977). These free radicals cause endothelial damage and dysfunction in the coronary arteries and cell injury within the tissue (Reimer *et al.* 1989), they disrupt the cell membrane proteins including receptors and ion channels (Kaminski *et al.* 2002). In comparison to the rat, humans treatment with fibrolytics within 2 hrs of ischaemia improves LV function and reduces the infarct (Mathey *et al.* 1985), occlusion for longer than 4-6 hours leads to irreversible cell necrosis. Consequently, the sooner thrombolytics are administered after the ischaemic episode the less better (Fenton 2006).

One form of cell injury that occurs within the myocardium post-MI is termed 'lethal injury' this occurs immediately after the on-set of ischeamia. Lethal injury is due to the decrease in cell ATP levels, due to attenuated oxygen and supply for ATP synthesis (Lazdunski *et al.* 1985). The depletion of ATP leads to the cessation of the Na^+/H^+ transporter pump and the Na^+-K^+ ATPase (Kusuoka *et al.* 1987). The consequence of this is a rise in intracellular Na^+, Ca^{2+} and a shift in cellular pH to a more acidic level. Sudden reperfusion after this has occurred in the ischaemic area results in the Na^+-K^+ ATPase and Na^+/H^+ transporter pump starting again but with the cellular concentration gradients disrupted and the osmolarity of the cell greatly increased the cell swells with fluid and bursts. The cells in the neighbouring non-infarcted myocardium are

subsequently damaged and die, through the cell-cell communication via gap junctions (Jennings *et al.* 1991). This immediate death is in comparison to cell death that occurs within hours and days of infarction. Cell death that occurs in the early remodelling period is likely to occur in the presence of an inflammatory response and occur through apoptosis and necrosis (Fliss *et al.* 1996).

Reperfusion prior to the critical period results in cellular damage extending from an energy demand that is not met by supply. Reversible cell damage, also referred to as 'myocardial stunning', causes a rapid decline in ventricular performance. Upon reperfusion ventricular performance slowly returns to normal and there is no permanent damage (Heyndrickx 2006). The period of stunning depends on the period of ischaemia, experimental models use 10-20 minutes in the rat myocardium to assess this phenomenon (Califf 1998; Heyndrickx 2006). During the period of stunning the myocardium is less responsive to ionotropic drugs.

Gender based differences in ischaemia reperfusion injury, support a protective role for $17\beta E_2$ (Guerra *et al.* 1999). Females exhibit a decreased inflammatory response and tissue injury as well as improved survival. In isolated buffer perfused rat hearts $17\beta E_2$ increases functional recovery through anti-oxidant effects and altered glucose metabolism (Beer *et al.* 2002). In canine models of ischaemia-reperfusion $17\beta E_2$ attenuates hydroxyl radical production, reperfusion arrhythmias, myocardial systolic dysfunction and temporary impairment of endothelial function (Kim *et al.* 1996; McHugh *et al.* 1998). Of the two ER expressed within cardiomyocytes, myofibroblasts and neutrophils, experimental models of ischaemia have not distinguished which receptor is responsible for mediating the protective effects of $17\beta E_2$. The recent development of selective agonists has increased our ability to determine the role of each receptor and understand the mechanisms of $17\beta E_2$ induced protection.

1.8. Cardiac rupture

Factors that delay and jeopardise the healing process after MI result in disrupted remodelling and even cardiac rupture. Other complications that may occur in the initial days after MI include re-infarction, pulmonary oedema and cardiogenic shock. Of these complication cardiac rupture has the greatest mortality rate. Cardiac rupture causes bleeding from the heart into the pericardium and ultimately cardiac tamponade (Dellborg *et al.* 1985; Reddy *et al.* 1989). The incidence of cardiac rupture is unpredictable and tends to affect middle-aged patients (> 65yearrs) who have no prior history of MI or angina. Additional risk factors include female gender and hypertension (Chandra *et al.* 1998; Lewis *et al.* 1969). The reasons for a higher mortality rate in females during the initial days post-infarction are not clear, but maybe due to differences in hormone levels and the response of vascular tissue to the initial insult. Figure 1.9 highlights the in-hospital mortality after MI in males and females, it's clear that mortality increases with age. The mortality rate is higher in females than in males until >80 years. The cause of mortality in this figure is not only cardiac rupture.

Cardiac rupture following MI occurs at an incidence rate of 2-7% yet it accounts for around 15% of all in-hospital mortalities post-MI and has a mortality rate over 90% (Becker *et al.* 1996; Reddy *et al.* 1989). The lack of treatment means that rupture is normally fatal. Surgery is the only possible intervention for cardiac rupture, it has a 27% mortality rate and the long-term survival rate after surgery is only 31%. Due to such a poor survival rate, a current lack of pharmacological treatment and the growing incidence of rupture following MI, it is paramount that a successful intervention is found. Clinically the most frequent form of rupture is LV free wall rupture (Dalrymple-Hay *et al.* 1998).

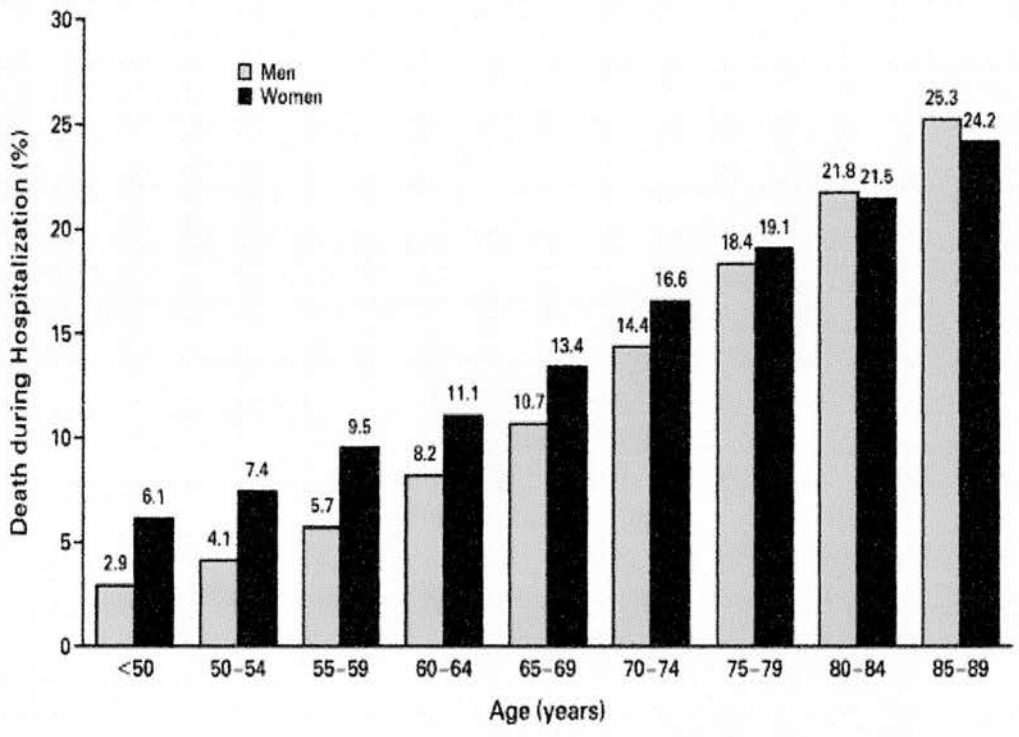


***Figure 1.9.* In hospital mortality after myocardial infarction.**
The mortality rate of humans according to sex and age during hospitalisation for myocardial infarction. Copyright permission granted from Vaccarino et al. (Vaccarino et al. 1999).

Rupture may occur within hours of the initial MI, approximately 50% of all cases occur almost instantaneously without the manifestation of an inflammatory response. In these cases cardiomyocyte apoptosis is likely to play a role in the defects that result in rupture (Reviewed by Wehrens *et al.* 2004). Rupture can also occur up to three weeks after the initial MI, although most cases of cardiac rupture in humans occur 3-5 days post MI. These later incidences of rupture occur in the presence of tissue necrosis and an inflammatory response (Solomon *et al.* 2002).

Following MI the myocardium undergoes a period of extensive remodelling. Inadequate cardiac remodelling and scar formation following MI is a frequent cause of cardiac rupture. Extensive myocardial expansion is correlated with an increased risk of

detrimental events such as rupture, aneurysms and long term heart failure (Pfeffer *et al.* 1990). After MI the acute increase in MMP collagenase activity, developing necrosis, oedema and inflammation degrades the matrix proteins and collagen. These processes result in a temporary decrease in structural support, deformed tissue architecture and wall thinning that may result in cardiac rupture. This period is sustained for 2-3 days, after which time new matrix proteins and collagens are synthesised and deposited. Consequently, the tensile strength is increased and the risk of rupture reduced (Reviewed by Creemers *et al.* 2001).

Only a small decrease in the collagen content of the ECM is necessary for rupture to occur, this is in contrast to an excess of collagen in the cardiac ECM, where a 2-3 fold increase can be buffered after which it may result in stiffness and mild dysfunction. In a canine model rapid-pacing is accompanied by diminished collagen content, which is then reversed during normal pacing, thus suggesting that the integrity of the myocardium is weakened by the abnormal pacing (Spinale *et al.* 1991). An increase in collagen content is associated with MI ensuring a stable scar to prevent cardiac rupture. Due to the narrow physiological range of collagen content in the ECM the components of the ECM must be tightly regulated, as described in Section 1.5.3.2, to prevent adverse pathological events (Ichihara *et al.* 2002; Jugdutt 2003). Experimental models of MI and cardiac rupture in the mouse have demonstrated the importance of MMPs in the remodelling process proceeding cardiac rupture (Heymans *et al.* 1999; Rohde *et al.* 1999). Rohde et al used a non-selective MMP inhibitor to demonstrate an attenuation of LV expansion after MI in mice (Rohde *et al.* 1999). More specifically Heymans et al have verified that an MMP-9 KO mouse has decreased incidence of cardiac rupture (Heymans *et al.* 1999)

Interestingly, younger women are more likely to die during the acute period after MI than their male counterparts (Vaccarino *et al.* 1999), some studies have attributed this to cardiac rupture (Hutchins *et al.* 2002; Nakano *et al.* 1985). The observation that this

difference is lost in older women suggests that sex hormones have an influential role in the mortality rates post-MI.

Experimentally in mice there is an increased mortality rate (Sharif 2002; van Eickels *et al.* 2003) and detrimental remodelling (Cavasin *et al.* 2004; Smith *et al.* 2000) in ovx females supplemented with $17\beta E_2$ compared to control treated animals. This finding suggests that E has a dual function within the CVS. The two fold properties of E seem to be a beneficial protective mechanism serving to inhibit the onset of CVD, but after the process of CVD has commenced in women E may have a detrimental role (Vaccarino *et al.* 1999). Despite studies in the mouse model of MI to investigate the mechanisms of detrimental remodelling the influence of $17\beta E_2$ has not been investigated.

1.9 Oestrogen and Hormone replacement studies

Since the 1940's E replacement therapy (ERT), comprised of conjugated equine E, has been prescribed to post-menopausal women for suppression of menopausal symptoms. The main reason for ERT use in this sub-section of the population is a reduction of menopausal symptoms such as an increased incidence of osteoporosis and hot flushes that result from decreased circulating E. In accordance with the known cardiovascular properties of E, unopposed ERT decreases the rate of atherosclerosis in women who have not had a previous coronary event (Hodis *et al.* 2001). Proudler et al have also noted that there is as much as a 20% decrease in angiotensin converting enzyme (ACE) activity in women taking ERT compared to post-menopausal women who are not (Proudler *et al.* 1995).

Unopposed E in ERT increases the risk of endometrial cancer and is therefore not appropriate for women who have an intact uterus. To circumvent the increased risk of endometrial cancer a progesterone analogue, such as medroxyprogesterone acetate (MPA) is incorporated into the ERT. Progesterone analogues have their own biological properties, among which are modifications of Es effects. The modifications to Es properties vary from acting synergistically, to having no influence at all or antagonizing

the properties of E (Simoncini *et al.* 2004a). Combined hormone replacement therapy (HRT) is now offered to women who have not previously undergone an ovariectomy, whilst ERT is offered to those who have.

MPA has mild androgenic and progesterone actions (Muddana *et al.* 2003). Progesterone receptors are expressed within VSMC and also within the heart (Ingegno *et al.* 1988; Knauthe *et al.* 1996). Stimulation of the vascular progesterone receptor by MPA is likely to have additional consequences on the E response, including adverse effects on circulating HDL and thrombolytic protein levels (Iruela-Arispe *et al.* 1996; The writing group for the PEPI trial 1995). Stimulation of the progesterone receptor inhibits vasorelaxation (Mercuro *et al.* 1999b) and in a mouse model progesterone increases vascular injury (Karas *et al.* 2001b).

In 1976 121,700 female nurses aged 30-55 years completed questionnaires on their hormonal status and medical history, including CVD for the 'Nurses' Health study' (Stampfer *et al.* 1985). The outcome of this study confirmed findings of the Framingham study (Feinleib 1976), demonstrating a positive correlation between ERT or HRT and a decreased risk and incidence of CVD. These and other observational studies (Reviewed by Herrington *et al.* 2003a) led to the concept that post-menopausal HRT could retain the apparent pre-menopausal cardioprotection. Results from clinically controlled primary and secondary prevention trials of hormone replacement, over 20 years later led to a dramatic rethinking of guidelines for the use of HRT in relation to cardiovascular risk.

The largest and most documented of these studies are the 'Women's Health Initiative' (WHI) primary prevention trial, which started in 1992, designed to study ERT and HRT for 8 years; and the 'Heart and Estrogen/progestin Replacement Study' (HERS), secondary prevention trial that started in 1993, designed to investigate the use of HRT for an average of 4.1 years.

The HERS study was the first study to publish controversial and unexpected results (Hulley *et al.* 1998). This study was designed to investigate HRT (0.626mg CEE and 2.5mg MPA) in post-menopausal women with pre-existing CVD. HERS looked at 2763 women with a mean age of 66.7 years and a subsequent HERS II study looked at the same women after 6.8 years. The surprising conclusion of HERS was a null effect of HRT on CVD, with an apparent detrimental effect of E in the first year and then an apparent reduction in the risk of CVD in years 3 to 5 (Hulley *et al.* 1998). The follow up study, HERS II concluded that the apparent beneficial effect of E seen in the 3rd to 5th years of the trial did not continue in subsequent years and consequently it was stopped with a conclusion that HRT should not be taken for prevention of secondary CHD (Grady *et al.* 2002).

The WHI was designed in 1991 to investigate ERT and HRT on health outcomes when used for primary prevention of CVD. The WHI enrolled 27,347 post-menopausal women aged 50-74 years (average age 63; 20% 70-79 and 12 years post-menopause) and randomised them to receive ERT (0.625mg CEE), HRT (0.625mg CEE and 2.5mg MPA) or placebo treatment. The WHI hit the headlines and gained media attention in 2002 when the combined HRT arm of the study was stopped prematurely after an average follow up of 5.2 years. The study was stopped due to an increased incidence of breast cancer (Rossouw *et al.* 2002; Steinberg *et al.* 1991). At this time point there was also a significant ($P<0.05$) increase in non-fatal MI and stroke in those taking HRT compared to placebo (Rossouw *et al.* 2002). Since then, in 2004, after almost 7 years the ERT arm of the WHI was also terminated due to an increased incidence of stroke and a null effect on CVD (Anderson *et al.* 2004).

The present advice from the 'American Heart Association', based principally on the outcomes of the WHI and HERS, is that ERT and HRT should not be used for primary or secondary prevention of CHD (Mosca *et al.* 2004). In addition, it has been recommended that women who choose to take ERT or HRT for other menopausal symptoms should have their CV risk assessed before commencing the treatment.

We do not know the effect that MPA has on the cardioprotective properties of E when given in HRT regimes. These clinical trials suggest that the progesterone analogue may inhibit E's beneficial properties and therefore attenuate any protection provided by E. The mechanisms behind this apparent attenuation within the myocardium are not known. A more accomplished and complete understanding of the pre-menopausal mechanisms of E both physiologically and pathophysiologically will enable post-menopausal E supplementation to be prescribed and managed correctly.

1.10. Study aims

The first aim of this thesis was to determine the ER responsible for the observed cardioprotection displayed by $17\beta E_2$ including neutrophil infiltration, oxidative stress and cell necrosis displayed by $17\beta E_2$. This was investigated using an experimental rat model of ischaemia-reperfusion with the use of a novel selective ER agonist and antagonist.

In light of the results from clinical trials on HRT the second aim of the thesis was to investigate the effect of MPA on the cardioprotective properties of $17\beta E_2$. To study the effect of MPA on the protective properties of $17\beta E_2$ we used an experimental rat model of ischaemia-reperfusion. After ischaemia-reperfusion cell necrosis and neutrophil infiltration were measured, and the impact of MPA on these measured.

The final aim of the thesis was to investigate the finding that despite the initial cardioprotective effects of $17\beta E_2$ there is a higher mortality rate during the acute remodelling phase of chronic MI. We aimed to explore the influence of $17\beta E_2$ on MMP and TIMP expression and activity post-MI in a mouse model. We also aimed to examine the effect of $17\beta E_2$ on neutrophil infiltration and cell death in this model.

CHAPTER 2

MATERIALS AND METHODS

2. Methods

2.1. Animals

All animal procedures were conducted and conform with the Guide for the Care of Laboratory Animals published by the US National Institutes of Health (NIH publication No 85-23, revised 1996) and in accordance with Home Office guidelines as outlined in the Animals (Scientific Procedures) Act 1988 under the project license 60/2813 and personal license 60/9013.The studies were approved by both the UK Home Office and the University of Edinburgh ethical review committee.

All animals were housed in a controlled environment of a 12 hour light/dark cycle (lights on 0700-1900 hrs) and a constant temperature of 21 $\pm$ 2°C and humidity. All animals were fed a standard laboratory diet composed of 75.09% carbohydrate, 14.38% protein, 2.71% oil and 0.25% salt (Special Diet Services (SDS), Witham, Essex, UK) and had free access to water.

Rats used were female Wistar (170-210g; Charles River, Margate, UK). Rats were allowed an acclimatisation period of one week in their new environment prior to commencing any surgical or experimental procedures. Mice used were C57bl6/129/SvJ (25-30g; Dr G.A.Gray's colony at the University of Edinburgh).

2.2. Surgical procedures and tissue collection

Animals were weighed prior to all surgical procedures. All surgery was carried out under aseptic conditions, skin clippers (Series 8900, WAHL, USA) were used to remove hair prior to surgery after which the area was swabbed with antiseptic wipes (Spontex, Professional discovery 1500). Animals were kept on a heating mat (37°C) to maintain body temperature throughout surgery. Buprenorphine hydroxychloride analgesic was administered subcutaneously prior to all recovery surgery (0.05mg.kg^{-1} and 0.5mg.kg^{-1} for mice and rats, respectively).

2.2.1. Recovery Surgery

2.2.1.1. Regulation of the oestrous cycle by bilateral ovariectomy

Circulating E levels vary throughout the oestrous cycle in female animals and this variation can cause problems with inconsistency within experiments. Controlling the circulating E levels overcomes this problem and inter-experiment variation. Control of the circulating E level is achieved through ovx to remove the ovaries, which are the primary organ responsible for E synthesis, and implantation of $17\beta E_2$ releasing pellets ($0.05mg.pellet^{-1}$, 21 day release, $50\text{-}100pg.ml^{-1}$, Innovative Science, Sarasota, USA).

Surgical techniques: Anaesthesia was induced either by inhalation of halothane (Halothane-vet, Merial Animal Health Ltd. UK) or isoflourane. Inhalation anaesthetic was administrated with a mixture of O_2 ($1.51\ min^{-1}$) and N_2O ($0.51\ min^{-1}$) for halothane or O_2 alone for isoflurane through a nasal cone after initially being induced in a chamber (4% induction and 1.7-2.5% maintenance for rats; 3% induction and 1.7-2% for mice).

The ovaries were exposed via an incision (5mm long in the mouse and 10mm long in the rat) in the dorsal flank and penetration of the abdominal cavity. The parovarian fat pad was identified and moved aside allowing the ovary and the associated oviduct and uterine horn to be exposed. A ligature (5/0 W581 Mersilk, Ethicon, UK) was secured tightly around the oviduct and ovarian vasculature prior to the ovary being severed. This procedure maintained haemostasis. The uterine horn was returned to the abdominal cavity and the incision closed. The abdominal wall was closed first with discontinuous sutures (5/0 W581 Mersilk, round bodied, Ethicon, UK), the skin was closed with 9mm metal wound clips (Reflex wound clips, Interfocus, Cambridgeshire, UK) in rats, and sutured in mice (5/0 W529 Mersilk, cutting, slim blade, Ethicon, UK). The animals were then randomly assigned to a treatment group and the appropriate pellet implanted subcutaneously at the nape of the neck between the scapula, this small incision was closed with sutures (5/0 W581 Mersilk, Ethicon, UK). The animals were then allowed to recover from anaesthesia. The animals were returned to cages once they were ambulant, they were caged with a maximum of three other animals, all having undergone the same

surgery. All animals were caged with animals receiving the same E treatment to prevent exposure to E through grooming of each other. Mice were then left for a minimum period of 7 days, and the rats were left for at least 14 days. During this period the animals were weighed regularly to ensure they maintained and gained weight.

Confirmation of E levels and activities: Body and uterine weights were recorded at the experimental endpoint to determine the influence of hormone supplementation. The implanted pellets achieve and maintain physiological E concentrations. Female mice and rats were randomised to receive either E or placebo pellets. E levels increase pre- and post- ovulation. E stimulates vascularisation and thickening of the endometrium through promotion of cells in the basal stroma of the uterus to enter mitosis (Frasor *et al.* 2003). In contrast at low E levels the endometrium is thin and atrophied, due to only the stratum basalis of the endometrium remaining. Consequently, the uterus weight can be used to confirm E status after ovx and as an indication of the circulating levels of E. At the experimental endpoint the uterus was removed and weighed (Figure 2.1 and Figure 2.2).

2.2.1.1.1. Detection of plasma oestrogen levels

Radioimmunoassay for $17\beta E_2$ was carried out to determine the plasma concentration of $17\beta E_2$ in ovariectomised mice and rats receiving placebo or $17\beta E_2$ pellets. Blood samples were collected by cardiac puncture, from placebo and $17\beta E_2$ treated group. The blood was mixed with 50µl (5000 $I.U.ml^{-1}$) of heparin the plasma was then separated by centrifugation at 4°C, 20,000g for 20 mins and stored at -20°C prior to assay. Plasma $17\beta E_2$ levels were measured using radioimmunoassay (MAIA RIA kit, Bio-Stat Diagnostic systems, Stockport, Cheshire, UK), as previously described (Glasier *et al.* 1989). $17\beta E_2$ extraction efficiency was greater than 90% and the detection limit of the assay was $5pg.ml^{-1}$. The intra- and inter-assay coefficients of variation were 4.0 and 15.8% respectively.

$17\beta E_2$ levels were typically within the levels found in the proestrous phase of the mouse oestrous cycle in those mice receiving $17\beta E_2$, whilst those receiving placebo had significantly lower plasma levels of $17\beta E_2$ (Table 2.1). In rats treated with $17\beta E_2$ plasma levels were in the middle of the physiological range (Table 2.1). The presence of low levels of $17\beta E_2$ in placebo treated mice and rats is most likely to be due to the other sites of production in the body after the ovaries have been removed.

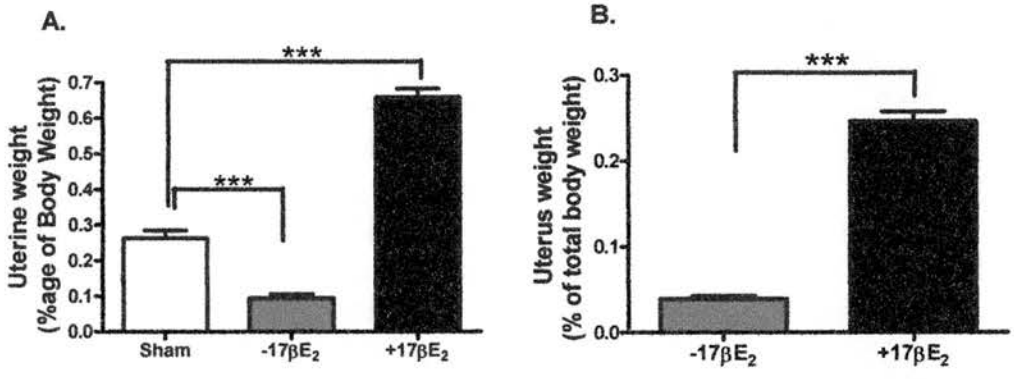


***Figure 2.1.* Effect of oestrogen status on uterine weight**
*Uterine weight of (**A.**) mice having undergone sham ovariectomy (n=10), ovariectomy and implantation of a placebo (n=16) or 17β-estradiol pellet (0.05mg/pellet; n=20) and (**B.**) of rats having undergone ovariectomy and implantation of either a placebo pellet (n=8) or 17β-estradiol pellet (0.05mg/pellet; n=9). Data is expressed as mean ± SEM and analysed using a One-way ANOVA followed by a Bonferroni's post-hoc test for the mouse data and a Student's unpaired two-tailed t-test for the rat data, ***P<0.001 compared to ovx and placebo treatment.*

Animal	Hormone Treatment	Plasma Estradiol level ($pg.ml^{-1}$)
Mouse	-17βE$_2$	42.56 ± 4.46
Mouse	+17βE$_2$	106.2 ± 3.19***
Rat	-17βE$_2$	20.68 ± 3.92
Rat	+17βE$_2$	61.42 ± 7.7*

***Table 2.1.* The effect of oestrogen status on the plasma estradiol levels in mice and rats.**
*Data is expressed as mean ± SEM and analysed using a Student's unpaired two-tailed t-test for the mouse and rat data respectively, *P<0.05; ***P<0.001 compared to ovx and placebo treatment.*

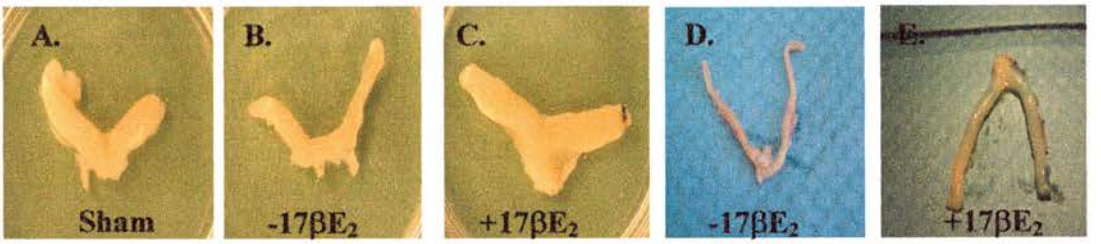


***Figure 2.2.* Oestrogen on uterine proliferation**
*Mouse uteri after (**A.**) sham ovariectomy or ovariectomy with (**B.**) placebo pellet or (**C.**) 17βE$_2$ pellet implanted. Rat uteri after ovariectomy with (**D.**) placebo or (**E.**) 17βE$_2$ pellet implanted.*

2.2.1.2. Mouse chronic coronary artery ligation

Ovx mice were anaesthetised by intraperitonial (i.p). injection of ketamine, xylazine, atropine (doses per 100g were 10mg, 2mg and 0.06mg) and prepared for surgery as described in Section 2.2. Once prepared for surgery the mice underwent endotracheal intubation with a blunted 12 G needle and were ventilated (HSE-Harvard MiniVent, Harvard Apparatus Ltd, Kent UK) with a mixture of room air and oxygen at 120 breaths-a-minute with a stroke volume of 200µl.

The eyes were coated with Lacri-lube® gel (Genusxpress, Aberdeen, UK) to prevent desiccation. Using a surgical microscope (Leica $MZ7_5$, Milton Keynes, UK) at 100x magnification a left thoracotomy 1cm long was performed via the fourth intercostal space. The exposed muscles were separated and tied back using a suture (5/0 W581 Mersilk, Ethicon, UK). This revealed the ribs and intercostal space, allowing the respiratory movement of the left lung and heart to be observed. The fourth intercostal space was confirmed by the curvature of the left lung, the rib spacing and the extra-thoracic emerging branch of the internal thoracic artery, which is visible at the fifth intercostal space. An incision was made in the fourth intercostal muscle, by lifting the fifth rib and making an initial puncture with blunt curved forceps. The forceps were then rotated and inserted into the chest cavity so that the long axis of their curve was laid inline with the ribs away from the heart. Spring scissors were used to cut through the intercostal muscle between the out-stretched forceps.

At this point the magnification of the dissection microscope was increased to 160x. A 1cm tissue clamp was inserted in the fourth intercostal space at the site of the incision. With the heart and medial border of the left lung visible the pericardium was gently disrupted enabling access to the heart. The pericardium was disrupted in a manner such that a sheet was laid over the lower left edge of the incision. The sheet was pulled more laterally to rotate the heart on its long axis as required to visualise the origin of the left coronary artery (LCA). Controlling the illumination through the microscope and elevating the left atrium using fine smooth tipped forceps, assisted identification of the

LCA. The LCA was typically found appearing beneath the left atrium at the atrial notch coursing towards the anterior aspect of the LV, with rapid pulsation of bright red blood visible in its walls.

A proline suture (6/0 W8271 Ethicon, UK) was inserted into the myocardium 2-3mm inferior to the intersection of LCA with the left atrial notch. The suture was passed beneath the artery but not into the ventricular cavity and brought out approximately 1mm medial to the insertion site. The suture was tied, the heart rhythm was immediately noted to alter and the ventricular myocardium seen to pale. The pericardium was replaced, the clamp removed and the intercostal space sutured (5/0 W581 Mersilk, Ethicon, UK) with discontinuous sutures. The Mersilk ties in the borders of the chest muscles were removed; the muscle borders were teased into their former positions. Negative pressure was created within the chest cavity with gentle pressure and this held the muscles in place over the 4^{th} intercostal space.

The skin was closed with continuous sutures (5/0 W581 Mersilk, Ethicon, UK). On completion of surgery animals received i.p atipamazole (AntiSedan, an α-adreno-receptor antagonist; $5mg.kg^{-1}$). Recovery sufficient for extubation was characterised by self-breathing movements and whisker twitching. At this time mice were transferred to a recovery cage with a heating pad, oxygen (delivered by funnel into which the mouse head was placed) and *ad lib* saline. The oxygen was removed once the animals were moving freely within the cage. The cage remained on a heat pad for the next 24-48 hrs. Control operated mice underwent the same surgical procedure, but the suture was passed underneath the LCA and removed rather than being tied to occlude the artery.

Tissue collection for necrosis, apoptosis and neutrophil infiltration analysis: Post-MI surgery the mice were re-anaesthetised as described above. The chest was opened and the heart removed from the chest cavity. The left atrium was removed and the remaining portion of heart was cut in half transversely from the apex to the base through the middle of the infarcted LV. Control hearts were cut in half transversely through the

LV in the same manner as the infarcted hearts (Figure 2.3). The heart was weighed and processed as described in Sections 2.3 and 2.4.2 for necrosis, apoptosis and neutrophil infiltration analysis respectively.

Tissue Collection for MMP and TIMP analysis: Post-MI surgery, the mice were anaesthetised as previously described in Section 2.2. Hearts from these mice were dissected into distinct areas, right ventricle (RV), non-infarcted LV, infarcted LV (I), infarct border (B) and the septum (S). Hearts from control, operated mice were dissected into areas representing those in the infarcted hearts.

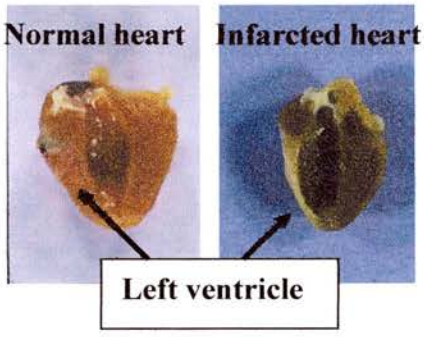


Figure 2.3. **Non-infarcted and infarcted heart post-MI.**
Mouse hearts cut transversely through the left ventricle infarct or the equivalent area. Thinning of the left ventricular free wall in the infarcted heart is clearly visible.

Tissue Explant Culture: The dissected areas were washed in Dulbecco's phosphate buffered saline without calcium and magnesium (DPBS; Gibco, Paisley, UK) supplemented with 80mg.l^{-1} gentamicin and 5mg.l^{-1} amphotericin B, then blotted dry and weighed. After a second wash the tissue was placed on a square of capillary matting in 1ml of tissue culture medium (RPMI 1640) containing antibiotics (50 IU penicillin, 50µg.ml^{-1} streptomycin), supplemented with insulin, transferrin (both 5µg.ml^{-1}) and sodium selenite (5ng.ml^{-1}). A comparable size of tissue from each section of the heart was maintained in culture at 37°C for 24 hrs in humidified air/5% CO_2. After the 24 hrs 900µl of culture medium was collected and stored at –20°C in 2ml Sarstedt tubes.

Freeze drying of culture medium: The Sarstedt tubes of culture medium were covered with Parafilm, the Parafilm had small holes punctured in it prior to being frozen at -70°C. Once the samples were thoroughly frozen they were placed on a freeze drier (EC Apparatus Thermo Life Sciences, Savant VLP 120 vacuum pump) and vacuum pump at 6-8 mbar pressure until dry (approximately 6hrs for 1ml), when the samples were dry the pressure was decreased. The samples were removed, the tubes capped and stored at -20°C. At the time of use this lyophilised culture medium was reconstituted in 100µl of dH_2O.

2.2.2. Non-Recovery Surgery

2.2.2.1. *In vivo* coronary artery ligation

Rats underwent ovx as previously described (Section 2.2.1.1) and anaesthetised. Anaesthesia was induced by i.p. injection of sodium pentabarbitone (60mg.kg^{-1}; Sagatal, Rhone Merieux, UK). A 2cm longtitudal incision was made in the anterior tracheal region, exposing the thymus glands. The thymus glands were separated allowing access to the jugular vein. The left jugular vein was isolated and permanently ligated at the proximal point, traction was added to the suture and it was secured to the table above the head, a second ligation was made approximately 5-8mm distal to the initial ligation. A small puncture was made in the vein and using a bent tipped 25G needle as a cannula guide the vein was cannulated with a heparinised saline filled cannula (100 IU.ml^{-1}; 0.75mm OD, Portex cannula, Harvard Apparatus, Kent, UK), for administration of anaesthetic (25mg.kg^{-1}; Sagatal, Rhone Merieux, UK).

The right carotid artery was located and isolated allowing the artery to be permanently ligated at the proximal end and then secured to the table above the head. A second ligature was placed 8-10mm lower and traction was added to this ligation with the use of needle holders. A further two ligations were placed in between the two most distal ligations. A small hole was made in the temporally occluded artery. With the use of a bent-tipped 25G syringe needle as a cannula guider the artery was cannulated with a heparinised saline filled cannula (100 IU.ml^{-1}; 0.75mm OD, Portex cannula, Harvard

Apparatus, UK). This cannula enabled the mean arterial blood pressure (MABP) to be recorded via a Maclab system (MacLab/4e, AD Instruments). The middle two ligations were secured prior to the traction being released from the most distal ligation enabling the cannula to be further advanced into the vessel. At this time the proximal suture was tied around the cannula and the middle two sutures were further secured.

The trachea was isolated and a ligature placed loosely around it prior to an incision being made allowing a cannula to be inserted for intubation (1.65mm OD, Portex cannula, Harvard Apparatus, UK). The rats were artificially ventilated with room air using a respirator (Harvard Rodent ventilator, model 683, Harvard Apparatus, UK) with the rate of 60 strokes.min^{-1} and a tidal volume of 1.5ml.100g^{-1}. This is sufficient to maintain PCO_2: 18-24mmHg; PO_2: 100-130mmHg and pH within physiological limits: 7.4 units (Clark *et al.* 1980). The body temperature was maintained at 37 $\pm$ 0.5°C with a thermostatically controlled under-blanket (Homeothermic Blanket Control Unit, Harvard Apparatus, UK).

After a short stabilisation period the LCA was ligated following a modified method previously described by Seyle (Selye *et al.* 1960). A skin incision was made over the thorax, the pectoral muscles were retracted to expose the thoracic wall. A thoracotomy incision was made at approximately 5mm to the left of the sternum by cutting through the 4th and 5th ribs, the pericardium was then cut to expose the heart. Gentle downward pressure was applied with forceps to either side of the incision resulting in the heart being exteriorised. A suture (5/0 Mersilk, round bodied, Ethicon, UK) was passed around the LCA just below the atrial appendage. The heart was immediately replaced into the thoracic cavity. A period of 20 mins was sufficient for the heart and all haemodynamic variables to stabilise, after which both ends of the ligature thread were passed through a short length of polythene tubing to form a reversible snare.

Regional myocardial ischaemia was induced through tightening the snare and securing the ligature to the workbench either side of the chest. Ischaemia could be visually confirmed by the altered appearance of the myocardium to a state of cyanosis. Previous work with radiolabelled microspheres has demonstrated that such a procedure reduces the blood flow to the area-at-risk by 98% (Toombs *et al.* 1992). Throughout ischaemia the MABP was recorded every 15 mins, an ECG was not recorded due to interference of the signal. After 45 mins of ischaemia the snare was released, and the myocardium allowed to reperfuse for 2 hrs. Throughout the reperfusion period the blood pressure was recorded at 30 min intervals.

Tissue collection: At the end of the reperfusion period the LCA was re-occluded at the same site and permanently tied. 1ml of Evans blue dye (Appendix 1.2i) was infused via the left jugular vein, enabling the perfused myocardium to be stained and identification of the area-at-risk (AAR; This protocol is illustrated in the diagram of Figure 2.5.). The Evans blue dye stains the non-ischaemic myocardium and leaves the ischaemic AAR pink and free from Evans blue (Figure 2.4). The heart was removed from the chest, snap frozen then stored at –20°C. To assess myeloperoxidase (MPO) activity the RV was removed after Evans blue perfusion. The remaining myocardium was separated into the AAR and the non-ischaemic area, based on the Evan's blue dye staining the non-ischaemic area. The AAR was sliced into 3-5mm slices weighed and stored at –20°C for a maximum of 14 days prior to analysis.

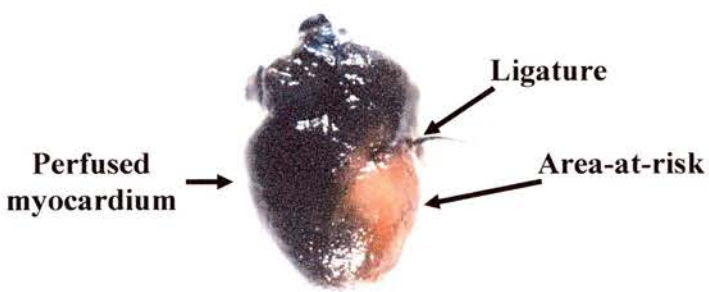


***Figure 2.4.* Ischaemia-reperfused heart dyed with Evans blue.**
A rat heart demonstrating the red AAR after ligation of the left coronary artery to induce ischaemia, and the blue perfused area of the heart that is not classified as at-risk.

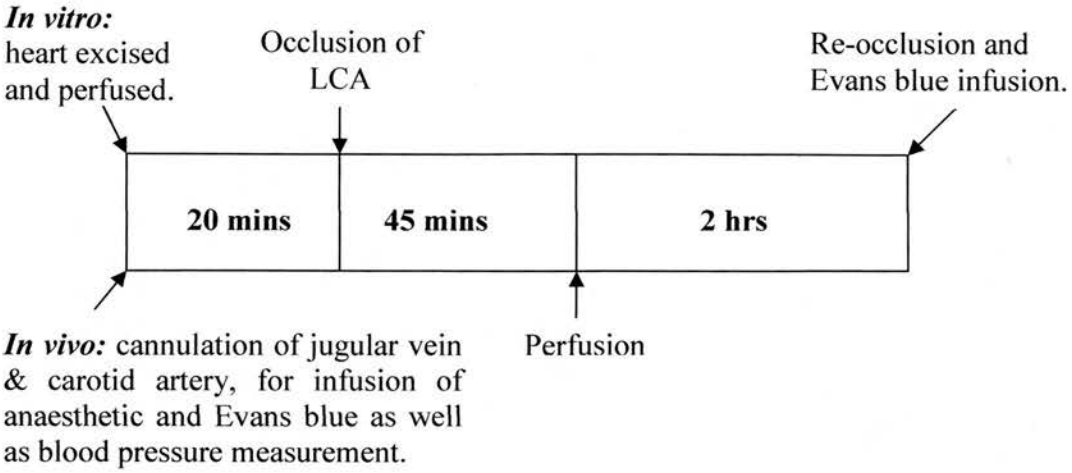


Figure 2.5. **Schematic diagram of the experimental protocol for ligation of the left coronary artery (LCA) *in vivo* and *in vitro*.**

2.2.2.2. *In vitro* coronary artery ligation.

The *in vitro* model of ischaemia-reperfusion lacks infiltrating blood during the entire protocol. Infiltrating neutrophils are therefore removed from the myocardium and it eliminates them as a source of the difference between the treatment groups, consequently making it clear to see if other protective mechanisms of E are through actions directly on the myocardium or through the infiltrating secondary mechanisms.

Rats underwent ovx and prepared as previously described in Section 2.2.1.1. At the time of surgery the rats were anaesthetised as described in section 2.2.2.1. The chest was opened along the sternum, exposing the entire chest cavity. Heparin sulphate (50IU; 50IU.ml^{-1}) was injected into the jugular vein to prevent thrombus formation in the coronary arterioles. A ligature (5/0 W595 Mersilk, round bodied, Ethicon, UK) was passed around the LCA prior to the heart being isolated. Upon removal from the chest the heart was placed in 50ml ice cold heparinsed Krebs (50 IU.ml^{-1}) Henseleit solution. The aorta was cannulated and the heart retrogradely perfused via the aorta on a modified Langendorff perfusion set up (Figure 2.6; Ferrari *et al.* 1996). Pre-filtered Krebs

Henseleit solution (Appendix 1.1) was perfused through the heart at a constant rate of 10ml.min^{-1} (Gilson, minipuls 3.0) and at a constant temperature of 37°C, gassed with 95% O_2/ 5% CO_2. The perfusate did not contain any hormones and therefore the experimental results were due to tissue retention and modifications from treatment prior to perfusion. After a stabilisation period of 20 mins the LCA was reversibly occluded for 45 mins. The ligature was passed through polythene tubing (Portex, OD 2.16mm, Harvard Apparatus, UK) and then clamped with a small vascular clamp. At the end of this ischaemic period the snare was released and the ischaemic zone re-perfused for 2 hrs. At the end of the reperfusion period the LCA was permanently occluded and 1ml of Evans blue dye infused via the injection arm to identify the AAR, as described in tissue collection of Section 2.2.2.1 (Figure 2.7). A schematic diagram of this experimental protocol is shown in Figure 2.5.

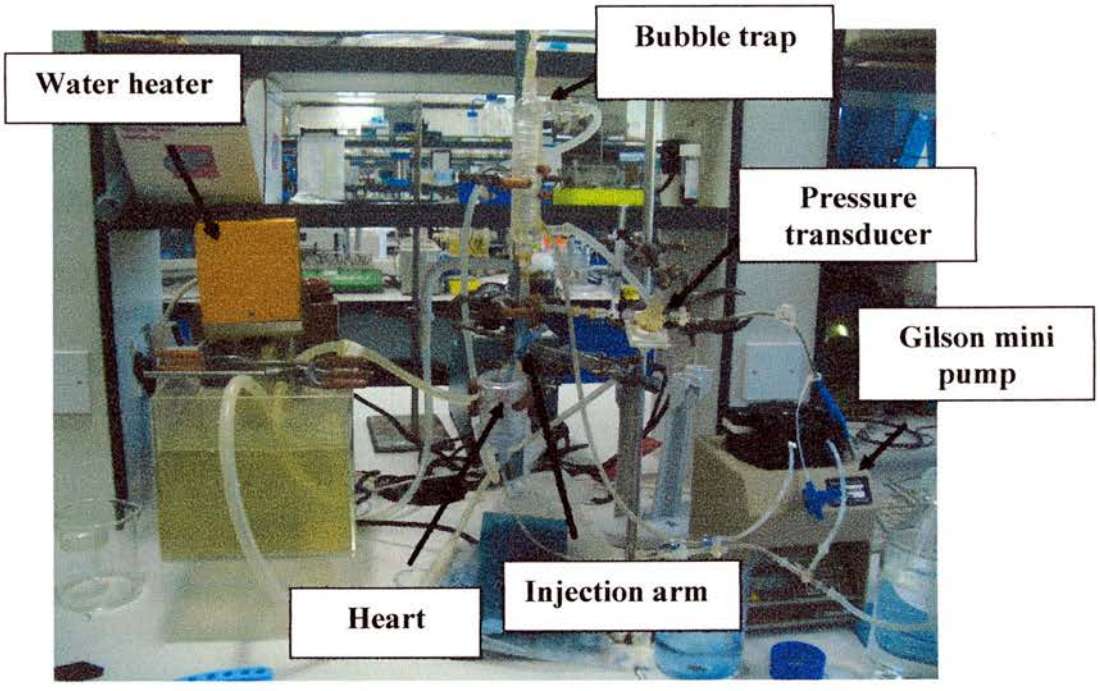


Figure 2.6. **Modified Langendorff perfusion set up**
Langendorff perfusion set up based on retrograde aortic perfusion of the isolated heart under constant flow conditions with Krebs-Henseleit.

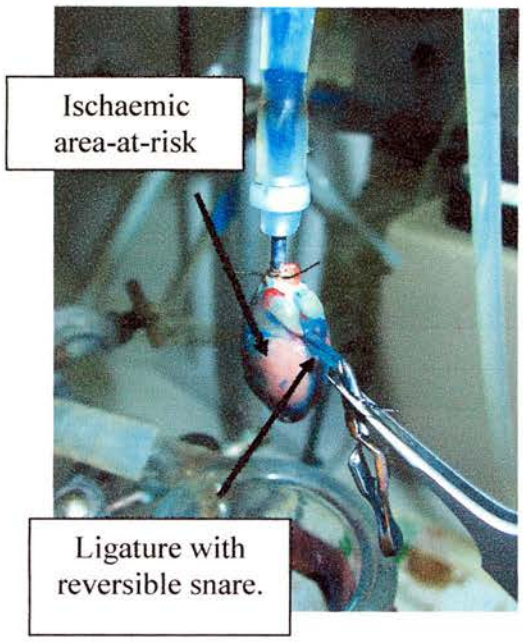


Figure 2.7 **Heart on the modified Langendorff perfusion set up with the left coronary artery ligated and Evans blue dye perfused.**
A typical rat myocardium having undergone acute ischaemia followed by reperfusion on the Langendorff perfusion set up. Evans blue dye stains the non-ischaemic area, whilst the pink zone beneath the ligature is the ischaemic zone.

2.3. Determination of infarct size and cell death

2.3.1. Area-at-risk

To assess the effect of hormone treatment on myocardial damage histochemical staining was used as an index of ischaemia-reperfusion injury. Myocardial injury was evaluated with triphenyltetrazolium chloride-Evans blue technique modified from Ytrehus (Ytrehus *et al.* 1994). In the presence of dehydrogenase enzymes in the non-necrotic AAR the 2,3,5-triphenyltetrazolium-chloride (TTC) is reduced to a bright red colour, whilst the necrotic area that lacks dehydrogenase activity remains white. These areas can

be differentiated from the non-ischaemic area of the heart that remains blue from the Evans blue dye (Figure 2.8).

Hearts that had been stored at –20°C were defrosted on ice and rinsed in 0.9% saline solution and cut into 2-3 mm slices from apex to base. The slices were then incubated at 37°C for 30 mins in TTC (Appendix 1.2ii). The slices of heart were fixed in 10% formalin (Appendix 1.2iii) for 15 mins and then rinsed in dH_2O allowing clearer area definition. The ischaemic AAR was separated from the non-ischaemic area. The AAR was then further divided into the necrotic and non-necrotic tissue. Each distinct area of myocardium was blotted dry and weighed. The total AAR was expressed as a percentage of the total heart weight and the necrotic area was expressed as a percentage of the AAR.

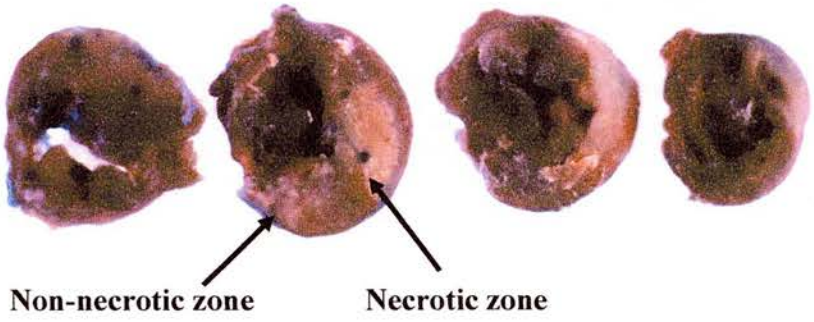


Figure 2.8. **Cross Sections of the rat myocardium.**

2.3.2. Apoptotic cell detection

Cell necrosis of damaged cardiomyocytes results from CAL, a method for detecting nectrotic tissue has been described in Section 2.3.1. Apoptosis through infiltrating leukocytes also occurs within the infarcted region, thus eliminating damaged cells. Apoptotic cells were measured and detected using a Deadend™ colorimetirc TUNEL system (Promega, Southampton, UK). This system is able to detect apoptotic cells at the single cell level by measuring the nuclear DNA fragmentation of cells, this is an

important marker of apoptosis in most cell types. The TUNEL system end labels the nucleotide fragments with a modified Tat-mediated dUTP Nick End Labelling (TUNEL) assay. A biotinylated nucleotide is incorporated at the 3' OH end of the DNA, this is then bound to streptavidin-HRP (horseradish-peroxidase) and detected using the peroxidase substrate hydrogen peroxidase and 3-3'-diaminobenzidine (DAB; Dakocytomation, Cambridgeshire, UK). The apoptotic nuclei stain a dark brown with this method. This system of detecting apoptosis is limited as it is unable to differentiate between necrotic and apoptotic cells, to confirm apoptotic cell death further studies utilising caspase 3 would be required.

Sections were prepared as described in Section 2.4.2.1. The manufacturers method was then followed for this assay.

2.4. Neutrophil infiltration measurement

2.4.1. Myeloperoxidase activity

2.4.1.1. Collection of control neutrophils

MPO is a lysosomal enzyme, predominantly stored in the azurophilic granules of neutrophils. Lysosomes of monocytes also contain MPO, this is about one third of the MPO present in neutrophils. The main role of MPO is that of a bacteriocidal agent, it works by catalysing the reaction of hydrogen peroxide (H_2O_2) and chloride ions into hydrochlorus acid thus destroying the bacteria. In addition to its bacteriocidal role it is thought to play a role in down-regulating the inflammatory response. As the predominant store of MPO is the azurophilic granules of neutrophils, it is a good indicator of neutrophil infiltration into the investigated area.

Neutrophils harvested from the peritoneal cavity of rats were used as a positive control for the MPO assay. The isolation of neutrophils was carried out according to a method previously described by Cunningham, with modifications (Cunningham *et al.* 1979). To collect neutrophils from the peritoneal cavity, 15ml of casein (sodium salt) in 0.9% NaCl was injected i.p into rats. This stimulates the accumulation of neutrophils within the

peritoneum. 16-18 hrs later the rats were sacrificed by anaesthetic overdose (100mg kg^{-1} Sagatal, Rhone, UK). 25ml of sterile phosphate buffered saline (PBS) containing 0.2% glucose and 20 $IU.ml^{-1}$ heparin was also injected i.p and massaged around to wash the peritoneal cavity. The peritoneum was then opened and the lavage fluid aspirated and collected in 50ml centrifuge tubes. The lavage fluid from each rat was kept separate and the volume of each tube increased to 50ml with PBS. The lavage fluid cell suspension was centrifuged for five mins at 400g and room temperature. The supernatent was discarded, any red blood cells remaining in the neutrophil pellet were lysed by resuspending the pellet in 10ml of ice cold 0.2% w/v NaCl solution for 20 seconds, after which 10ml of ice cold 1.6% w/v NaCl was added to return the cells to isotonic conditions. The cells were then centrifuged for 5 mins at 400g and room temperature, the supernatant was discarded. The cells were washed with 50 ml PBS containing 0.1% bovine serum albumin (BSA), to maintain their viability and decrease aggregation and then centrifuged at 400g at room temperature for 5 mins. The supernatant was discarded prior to a second wash of 10ml 0.1% BSA in PBS, this was centrifuged for 5 mins at 400g room temperature. The resulting pellet of neutrophils was then stored at –20°C until required for assaying.

2.4.1.2 Myeloperoxidase activity assay

MPO extraction from isolated peritoneal neutrophils and heart tissue was carried out according to a modification of the protocol previously described by Williams (Williams *et al.* 1994). Briefly, the AAR or neutrophil suspension was homogenised (T8 Ultra-turrax, Scienific Laboratory Supplies, UK) in 0.02M phosphate buffer (Appendix 1.3i; $50mg.ml^{-1}$). The homogenate was centrifuged at 10,000g for 15 mins at 4°C. Centrifugation caused cell lysis but left the MPO granule intact and thus removed other peroxidases such as haemoglobin peroxidase. The supernatant was discarded and the pellet resuspended in 0.05M phosphate buffer containing 0.5% hexacyltrimethylammonium bromide (HTAB; Appendix 1.3ii; $100mg.ml^{-1}$). The samples were then briefly homogenised for a second time. The HTAB is a detergent that lyses the azurophilic granule consequently causing the release of MPO from the

granules. The resulting homogenate was then taken through 4 cycles of freeze-thaw followed by a 2 hr incubation at 60°C. MPO is heat stable, this property is exploited here to eliminate inhibitory factors within the tissue which can mask the MPO activity. After the 2 hr incubation a 10 second sonication was followed by a final centrifuge at 10,000g and 4°C for 15 mins.

At the time of analysis for MPO activity the control samples (obtained as described in Section 2.4.1.1) were thawed on ice and assayed. 30µl of the sample supernatent was aliquoted into a 96 well plate and mixed with 200µl reaction buffer (Appendix 1.3iii). The absorbance was measured at a wavelength of 405nm (Thermo Labsystems; multiskan Ascent), over a period of 10 mins. A standard curve was constructed using MPO from human leukocytes in sodium acetate 0.02M. MPO activity was defined as the quantity of enzyme degrading 1µmol of peroxide.min^{-1} at 25°C expressed in units per g weight (U.g tissue^{-1}).

2.4.2. Immuno staining and histochemistry

2.4.2.1. Tissue Preparation

After harvesting the organs they were fixed in 10% neutral buffered formalin for 24 hrs. This fixative is a noncoagulant and therefore does not alter the fine network of tissue proteins and results in clear histological detail upon staining. The tissue was then transferred to 50% ethanol to remove the formalin and stored in 50% ethanol until processing.

During processing the tissue was passed through a series of increasing concentrations of ethanol to remove any resident water from between the protein network. The tissue was then immersed in xylene, which removes ethyl alcohol from the tissues, which is immiscible with paraffin. Finally the tissue was infiltrated with melted paraffin that provides a supporting matrix for the tissue.

The wax blocks with the hearts embedded were then cut into 5μm sections using a microtome. Creases and folds were eliminated from the sections by floating the sections out on a water bath (40°C). The water bath also assisted the transfer of the sections onto TESPA (3-Aminopropyltriethoxy-saline; Appendix 1.8) coated or electrically charged microscope slides (BDH Laboratory Supplies, Dorset, UK). The sections were air dried and then incubated at 37°C overnight to assist adherence. Prior to using the sections for any analysis they were treated by submersion in xylene (2 x 5 mins) to remove the wax. The sections were then re-hydrated through decreasing concentrations of alcohol (100, 95, 70%, 2 mins each) to water.

2.4.2.2. Histological analysis by Haematoxylin and Eosin Stain

Haematoxylin and eosin is a general stain used to identify the cells present within a section. The haematoxylin is a basic stain and therefore stains basophilic structures, such as chromatin within the nucleus. In comparison eosin is an acidic stain and consequently stains acidophilic structures, including collagen, muscle filaments and mitochondria.

The sections were prepared as described in Section 2.4.2.1 and then immersed in water for 5 mins. The slides were passed through Harris's haematoxylin for 2 mins and rinsed in tap water prior to 5 seconds in acid alcohol (Appendix 1.4i). The slides were then rinsed in tap water again and immersed in Sotts tap water (Appendix 1.4ii). This procedure differentiates the staining, the acid alcohol removes a uniform layer of haematoxylin stain from the cell whilst the Scotts tap water ensures that the haematoxylin stains the nucleus a distinct blue. After the Scotts tap water the slides were briefly exposed to 100% ethanol prior to staining with eosin (0.5% alcohol solution) for 5 mins. The sections were finally washed in tap water and dehydrated through the ethanol gradient and finally in xylene before mounting coverslips with DPX mount.

The sections were viewed under a light microscope (Ziess, Welwyn Garden City, UK) for the detection of neutrophils and macrophages. The leukocytes could be differentiated due to their distinct morphological differences. The neutrophils have a diameter of 6-

8μm and a multi-lobed nucleus that stains blue with haematoxylin compared to the pink cytoplasm with eosin. The neutrophils are differentiated from the macrophages which are larger, typically 9-12μm in diameter, the cytoplasm stains a darker pink with a kidney shaped nucleus that is eccentrically situated.

2.4.2.3. Immunohistochemistry

With the paraffin wax removed as described in Section 2.4.2.1, the sections were incubated for 30 mins in 3% H_2O_2. Incubation with this peroxide removes the endogenous peroxidases from within the tissue. The slides were then washed twice in Tris buffered saline (TBS) for 5 mins and treated to block endogenous biotin activity. Endogenous biotin activity was blocked by a 30 min incubation at room temperature with blocking serum (Table 2.2). After this incubation the excess blocking serum was removed and the sections were incubated at 4°C overnight with primary antibody, diluted in blocking serum (Table 2.2).

The following day, the primary antibody was washed off with TBS and the secondary antibody was added to the sections. Sections were incubated for 30 mins at room temperature in the biotinylated secondary antibody (Table 2.2). The secondary antibody was washed off in TBS to remove the excess unbound antibody. A 30 min incubation at room temperature with ABC-HRP (Dakocytomation, UK) followed, this binds to the biotinylated secondary antibody for detection. Sections were then washed in TBS again and bound secondary antibody was visualised using DAB (Dakocytomation, UK), this substrate reacts with the HRP to produce a brown stain, the colour change was monitored microscopically. DAB is active for up to 20 mins, it was inactivated once staining was apparent by washing the sections in water. The tissues were then counterstained with haematoxylin, and mounted using pertex.

GR-1 was used as a histochemical marker for neutrophils. GR-1 is a myeloid differentiation antigen, it is a glycosyl-phosphatidylinositol linked protein thus it remains attached to the cytoplasmic membrane and can be released by a phospholipase.

GR-1 is expressed in bone marrow of cells from a myeloid linage; the expression is regulated through development. Monocytes express GR-1 transiently during their development in bone marrow, however neutrophils express GR-1 both during development in bone marrow and once matured in the periphery. This expression of GR-1 makes it a good marker for neutrophils. Haematoxylin staining enabled the presence of neutrophils to be confirmed through co-staining the multi-lobed cell nucleus with the GR-1.

Once stained the sections were analysed and photographed at 1000x magnification using a laboratory microscope (Olympus BH-2 light microscope; Olympus Optical, London, UK) fitted with a prior automatic stage (Prior Scientific Instruments Ltd., Cambridge, UK). Neutrophil counting was performed with the use of Image-Pro Plus 4.5 with Stereologer-Pro 5 plug-in software (Media Cybernetics UK, Wokingham, Berkshire, UK). Ten points within the infarcted region of each heart were chosen at random by the computer, the number of neutrophils within these areas was averaged for each heart section. The same procedure was carried out in the RV as an internal control. In control operated hearts an equivalent section on the LV was selected for comparison to the infarcted LV.

Application	Antibody	Blocking Serum	Primary antibody	Secondary antibody
Immunohistochemistry	GR-1 Ebioscience (14-5931-82) 1:200 (2.5μg/ml)	Rabbit in TBS 5% BSA	Rat-anti-**mouse**	Rabbit-anti-rat (mouse absorbed) biotinylated Vector Labs 1:500
Western Blot	MMP-13 Chemicon (MAB13426) 1:1000 (0.2μg/ml)	5% BSA in TTBS	Mouse-anti-**rat**	Sheep-anti-mouse Horse-raddishperoxidase Amersham 1:5000
Western Blot	TIMP-2 Chemicon (AB19078) 1:1000 (1μg/ml)	5% BSA in TTBS	Rabbit-anti-**human**	Donkey-anti-rabbit Horse-raddishperoxidase Amersham 1:2000

Table 2.1. **Antibodies used for detection of expression of neutrophils, MMP-13 and TIMP-2 by immunohistochemistry and western blots respectively.**

The primary and secondary antibodies, with dilutions, and the blocking serum used for the detection of neutrophils, MMP-13 and TIMP-2 in the mouse myocardium after coronary artery ligation. BSA-bovine serum albumin; TTBS-Tween 20 tris buffered saline.

2.5. MMP and TIMP expression and activity

2.5.1. SDS PAGE

SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) gels were used to detect secreted MMP expression and activity as well as TIMP activity post-MI in the mouse myocardium.

The PAGE gel is formed by the polymerisation of acrylamide monomers into long chains and cross-linking these chains using *N,N'I*-methylene bisacrylamide. The polymerisation is initiated by free radicals produced from ammonium persulphate (APS) in the presence of TEMED (*N,N,N,N'*-tetramethylethylenediamine). The gel solution was degassed to remove oxygen prior to adding the TEMED. Degassing the solution enabled the production of reproducible gels because atmospheric oxygen scavenges the free radicals and therefore inhibits polymerisation. SDS is an ionic detergent used to dissociate the protein into individual peptides. The SDS saturates the peptides with a negative charge so that all the peptides of the same length migrate with a uniform negative charge during electrophoresis. The percentage of the gel refers to the acrylamide-bis, this determines the pore size and density in the gel for the peptide migration. A higher percentage of acrylamide-bis results in smaller pores and therefore greater separation of smaller molecules. The APS is very hygroscopic and begins to decay as soon as it is in solution with water, for this reason it was made up fresh every morning.

The BioRad mini system (Hertfordshire, UK) with a discontinuous electrophoresis system was used. The resolving gel was made up and allowed to polymerise with a layer of sec-butanol (Appendix 1.6i) on top, this excluded oxygen from the surface. 7.5% or 12% $w.v^{-1}$ SDS-PAGE gels were used as resolving gels. The stacking gel was made up on the morning the gel was being run in the same way as the resolving gel (Appendix 1.7ii, iv and vi).

Prior to being loaded on to the gels the samples were mixed with an equal volume of sample application buffer (Appendix 1.5i and 1.6ii). The sample application buffer does not alter the sample but allows the migration of the sample to be identified and traced. Molecular weight markers were always loaded in to the final lane of the gel so that proteins of unknown weight could be determined, whilst those of a known weight could be identified. Broad range markers (~30-120 kDa; BioRad, UK) were loaded on to 7.5% gels whilst low range markers (~20-120 kDa; BioRad, UK) were loaded onto gels of 12%. The molecular markers also enabled the separation to be visualised, ensuring that the current had passed through the gel sufficiently. Once the samples were loaded onto the gel, a constant voltage (100V) was passed through the gel until the dye front had just reached the end of the gel.

2.5.2. Densitometric analysis

Once scanned the gels and blots were analysed using 'Quantity one' (BioRad software, UK) allowing quantitative analysis of the density of bands and therefore the protein expression or activity. The same area was used to analyse the density of bands in all the gels. This allowed for comparisons of density between samples within each gel. Within each gel a background reading was taken and subtracted from all the other readings, the background reading was taken from in between lanes or at the edge where nothing had been loaded onto the gel or transferred onto the membrane. Subtracting the background eliminated the variance between gels, which allowed different gels to be collated and compared.

2.5.3 Protein expression by Western blotting

Western blots were used to detect protein expression within the samples, collected as described in Section 2.2.1.2. Once separated through electrophoresis the proteins bind to the membrane, which immobilizes them and thus makes them accessible to detection with specific antibodies.

Once mixed with application buffer (Appendix 1.6ii) the samples were passed through reducing conditions (boiled for 5 mins and then centrifuged at 13,000g for 3 mins at 4°C) before being loaded onto the gel. The reducing conditions meant that the conformation of the proteins passing through the gel were of a different shape and conformation, for example the disulphide bonds were broken. This conformational shape change results in a slight change in the molecular weight of the protein observed.

After approximately 2 hrs electrophoresis in tank running buffer (Appendix 1.6iii) when the dye front had run off the gel, electrophoresis was stopped. The gel was then equilibrated in transfer buffer (Appendix 1.6iv). This buffer was used to transfer the separated proteins from the gel onto a nitrocellulose membrane. During tank transfer the proteins are eluted from the gel and absorbed by the nitrocellulose membrane, the proteins then cross link onto the membrane making them accessible for the antibody to bind to. The transfer was set up so that the gel and membrane were sandwiched next to each other between fibre pads and filter paper and then a continuous current of 100V passed through it for 1 hr in transfer buffer (Appendix 1.6iv). When making the sandwich of gel and membrane up prior to transfer, the molecular weight markers were marked on the membrane. The markers were visible through the membrane, marking them with a pin prick on the membrane ensured that their position on the membrane was known if the marker bands did not transfer correctly.

After the transfer of proteins onto the nitrocellulose membrane the membrane was blocked in blocking solution (Appendix 2) for 1 hr. The membrane was then exposed to the specific primary antibody (Appendix 2) of interest for 2-2.5 hrs. Following this incubation there was a wash cycle of 4 x 5 min washes and one 15 min wash in wash buffer (Appendix 1.6vi) before incubation with the secondary HRP linked antibody (Appendix 2). After a 1 hr incubation with the secondary antibody the membrane went through a second wash cycle as above. The presence of proteins on the membrane was detected using the 'enhanced chemiluminesence' (ECL) method. This detection is based on the luminescent properties of luminol. HRP labelled antibody bound to the protein of

interest on the nitrocellulose membrane catalyses the oxidation and excitation of luminol. Excited luminol decays with a $t_{1/2}$ of 60 mins. As lumionol returns to its ground state it emits light, this light is detected by exposing the membrane to photographic film, this results in stronger dark bands at sights where there is more protein present. After ECL detection the nitrocellulose membrane was incubated for 45 mins in stripping solution (Appendix 1.6viii), to remove the antibodies from the membrane. The resulting photographic film was scanned into the computer and analysed as described in Section 2.5.2.

Non-specific binding (NSB) was determined by incubating the nitrocellulose membrane with blocking solution in place of the specific primary antibody. All primary antibodies were diluted in 5% BSA-TTBS, 0.025% sodium azide (Appendix 1.6vii). Secondary antibodies were diluted in TTBS.

2.5.3.1. MMP-13

Aliquots of the sample (15µl) were loaded on to a 7.5% SDS-PAGE gel. Due to the use of a primary antibody raised in mouse against mouse there was a lot of NSB detected (Figure 2.9a). The high back-ground NSB detected was due to other proteins within the mouse genome that have a similar sequence to the specific MMP-13 sequence that the antibody is designed to detect. To overcome the problem of excessive NSB masking the result an additional 1 hr incubation prior to the primary antibody incubation was needed. This additional incubation was with a 'mouse-on-mouse' (MOM) block (Vector labs, Peterborough, UK), this additional block reduced masking of results by NSB (Figure 2.9b). This primary antibody detected MMP-13 protein as a band at ~60 kDa and 48 kDa, the latent and active proteins respectively.

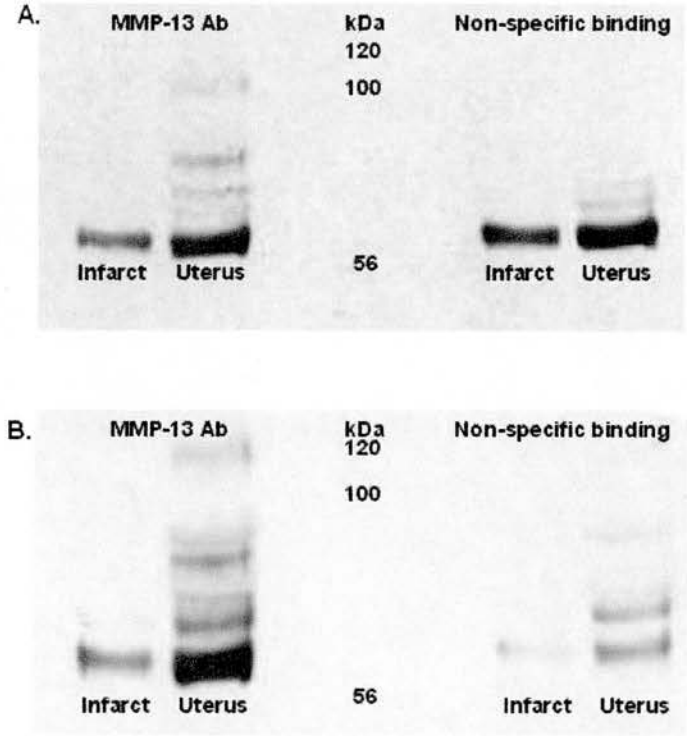


***Figure 2.9* Western Blot of MMP-13**
The blot, using ECL detection ***(a.)*** *without an additional incubation in MOM block and* ***(b.)*** *with an additional incubation in MOM block.*

2.5.3.2. TIMP-2

Aliquots of the samples (50µl) were freeze dried, as described at the end of Section 2.2.1.2. This freeze drying and reconstitution in dH_2O allowed for an equivalent of 50µl to be loaded onto the gel. The samples that were loaded onto the gel were concentrated 2.5x. The expression of TIMP-2 was detected on a 12% SDS-PAGE gel. TIMP-2 is seen as a band at ~26 kDa, there is a small shift in size from that detected in the reverse zymography, this is due to the reducing conditions in the Western blot compared to the non-reducing in the reverse zymography.

2.5.4. TIMP Activity by Reverse Zymography

Secreted TIMP activity was assessed by reverse zymography (Riley *et al.* 1999). Reverse gelatin zymography identifies TIMP-1, -2, -3 and –4, however TIMP-1, glyosylated TIMP-3 and TIMP-4 have similar molecular weights (28 kDa, 27 kDa and 30 kDa respectively) that cannot be discriminated against in the separation. The gel contained gelatin ($1mg.ml^{-1}$) and a cell cultured medium containing MMP-2 activity (from BHK-21 cells which constitutively secrete MMP-2; University Technologies Inc.), therefore at the discrete sites of TIMP activity the MMP-2 within the gel was inhibited and the gelatin not degraded, producing discrete dark bands.

An initial spectrum of secreted TIMP activity throughout all the regions of the heart was obtained, from this specific regions thought to be of most relevance and importance to LV remodelling were selected for reverse zymography. The chosen areas of myocardium were the RV, the I area and the B zone. The I and B are the two areas where most structural change and thinning is observed in the heart during MI and the RV remains unaltered during infarction of the LV and is therefore a good internal control.

The original samples (as prepared in Section 2.2.1.2) were concentrated; 20µl aliquots were freeze-dried, as described in Section 2.2.1.2 then reconstituted in dH_2O. This allowed a volume that was 3.3x concentrated equivalent to 20µl to be loaded on to the gel. The gels were subjected to electrophoresis in running tank buffer (Appendix 1.5ii) for approximately 2 hrs as described in Section 2.5.1. The gels were washed for 2 hrs at room temperature in wash buffer (Appendix 1.5iv) prior to an 18 hr digestion period in digestion buffer (Appendix 1.5vii) at 37°C. Following digestion the gels were stained for 3 hrs in 0.5% Coomassie blue R250. This was followed by approximately 2 hrs in de-stain solution (Appendix 1.5viii) to reveal dark bands of protein compared to the lighter areas where the substrate within the gel had been digested. A sample of human amniotic fluid collected at term in active labour and characterised previously was used as a positive control (Riley *et al.* 1999). The mouse TIMP-1, unglycosylated TIMP-3 and

TIMP-4 were seen as one band at approximately 30 kDa. TIMP-2 exhibited a molecular weight of 21 kDa in the non-reducing conditions of reverse zymography and unglycosylated TIMP-3 was seen as a discrete band at 21 kDa. The resulting gels were rinsed in dH_2O and then scanned into the computer and analysed as described in Section 2.5.2.

2.5.5. MMP activity by Gelatin Zymography

The activities of MMP-2 and MMP-9 secreted within the myocardium were assayed by gelatin substrate zymography (Riley *et al.* 2001). Activity of MMP-2 and MMP-9 was detected using 10µl aliquots of the sample (as prepared in Section 2.2.1.2) on an SDS-PAGE gel containing gelatin ($1mg.ml^{-1}$: Appendix 1.7i).

The samples were separated by electrophoresis for approximately 1hr in running tank buffer (Appendix 1.5ii), this was visually assessed by the dye front reaching the end the gel. The gels were then washed in Triton wash buffer (Appendix 1.5iii) and incubated in digestion buffer (Appendix 1.5iv) for 18 hrs at 37°C. After the incubation in digestion buffer the gels were stained in Coomaise blue R250 for 3 hrs and finally destained as described previously in Section 2.5.4. This process revealed discrete regions where the gelatin had been degraded by the MMP gelatinase activity. A sample of human amniotic fluid collected at term in active labour was used as both a positive and comparative control. The human amniotic fluid has previously been characterised in the laboratory (Riley *et al.* 1999). The resulting gels were rinsed in dH_2O and then scanned into the computer and analysed as described in Section 2.5.2.

2.6 Oxidative stress detection by Electron Paramagnetic Resonance

Oxidative stress is caused by reactive oxygen species, which include superoxide, singlet oxygen, peroxynitrite and H_2O_2. It is defined as an imbalance between pro-oxidants and anti-oxidants, with the former prevailing.

Detecting oxidative stress through the measurement of free radicals provides a clear indication of the oxidative stress. Electron Paramagnetic Resonance (EPR) enables the detection of free radicals. There are two approaches to EPR spectroscopy, they differ in their method of stabilising the reactive free radical species long enough to be detected (Vergely *et al.* 2003). The first of these methods is a 'freezing technique' where the tissue is snap-frozen in liquid nitrogen. The freezing technique was largely developed by Zweier *et al.*, when investigating free radical generation in *in vitro* perfused post-ischaemic rabbit hearts (Zweier 1988). This revealed an increased production of oxygen-derived radicals following reperfusion and demonstrated the functionality of EPR in recording this increase in free radicals. The freezing technique is however limited because it only provides information on what is happening at a chosen time. The second method of stabilising the reactive free radicals is through the use of spin-traps. The development of spin-traps, molecules that react with free radicals to produce a stable adduct which can then be detected by EPR, has provided an opportunity to investigate the time course of radical production continuously. This technique has previously been used to detect the brief oxidative burst that occurs in the first two minutes following ischaemia-reperfusion *in vitro* isolated buffer perfused hearts (Wang *et al.* 1996; Zweier *et al.* 1989).

EPR was used to detect and quantify the free radicals produced in a given sample. The free radicals, which we were interested in are generally transient due to a very short half live, for example superoxide has a half life of milliseconds and therefore difficult to detect using instrumental analysis. EPR is able to detect the free radicals through the formation of a more persistent radical, allowing the concentration of persistent radicals

to accumulate. Persistent radicals can be formed from these transient free radicals through a reaction with a spin-trap. The spin-traps used in this work are nitrogen centred compounds based on hydroxylamines. Free radicals react with this to form the more stable nitrogen centred adducts. During EPR measurement, the sample containing the stable 'adduct' was placed in a magnetic field where it was irradiated with microwave energy of a known wavelength.

During this study the 1-hydroxy-3-carboxy-pyrrolidine (CP-H; Axxora, Ltd, Nottingham, UK) spin-trap was used in EDTA solution. EDTA was necessary to counter the effect of certain metal ions. CP-H is cell permeable, non-toxic and forms adducts with the main free radicals involved in this study. CP-H also has a degree of resistance to signal break down by endogenous antioxidants. In a sub set of experiments SOD was also mixed with CP-H. The presence of SOD eliminates superoxide free radicals predominantly produced by the neutrophils.

The EPR readings were recorded using the Magnettech MiniScope MS 200 which operates in the X-Band Microwave frequency at approximately 9.5GHz, with MiniScope Control 6.51 software. The EPR parameters used throughout were set as follows; B0 Field – 3356G, Sweep – 44G, Sweep Time – 30s, No. of passes – 1, Modulation Amplitude – 1500mG, Microwave Power – 20mW.

After *in vivo* ischaemia-reperfusion of the rat myocardium as described in Section 2.2.1, 50IU of heparin was injected into the vena cava. The myocardium was removed into 50ml heparinised saline (50 $IU.ml^{-1}$) prior to being retrogradely perfused via the aorta on a Langendorff perfusion set up, as described in Section 2.2.2.2. The heart was allowed a stabilisation period of 20 mins prior to the CP-H spin-trap (10µl; 0.1M in EDTA, 0.01M to give an excess of spin trap) being injected into the perfusate above the heart through the injection arm of the Langendorff perfusion set up (Figure 2.6). 500µl of perfusate (drops 6-11 after the injection of spin-trap) was then collected from the base of the heart and incubated for 25 mins at 37°C before recording the signal produced from free

radicals with EPR. This procedure was repeated 4 times, with 2 mins between each sample collection. Spin-trap was firstly perfused through the myocardium with the LCA occluded by the formation of a reversible snare (as described in Section 2.2.2.2), therefore only the non-ischaemic myocardium was perfused. After 4 samples had been collected from the non-ischaemic myocardium the reversible snare was released and the ischaemic area perfused. The procedure of injecting spin-trap into the perfusate above the heart and collecting 500μl of perfusate from the base of the heart was then repeated. Perfusing the entire heart enabled the free radicals produced from the entire heart including the ischaemic AAR to be measured and compared to the free radicals produced from the non-ischaemic myocardium when the LCA was occluded.

In some experiments (n= 8) SOD was pre-mixed with CP-H prior to injecting it above the heart (50μl of 5000U SOD with 10μl CP-H per sample) to assess the contribution of superoxide radicals to the total radicals measured. SOD was only perfused through the entire heart. All samples were compared to and corrected for the auto-oxidation of the spin-trap in Krebs-Henseleit buffer, 500μl of Krebs-Henseleit buffer was collected from the base of the heart and incubated with CP-H in the same fashion as the samples. After all the perfusate samples had been collected, the LCA was permanently occluded and Evans blue dye was perfused. The myocardium was then stored and analysed as described in Section 2.2.2.2 and 2.3.1 respectively.

2.7. Statistical analysis

All statistical analysis was carried out using Graphpad Prism. Where appropriate a one-way ANOVA, two-way ANOVA or unpaired or paired two-tailed students t test and Bonferroni post-hoc test was used to compare mean values between groups. Kaplan Meier survival plots were analysed with a logrank test. All data is expressed as mean $\pm$ SEM with n indicating the number of animals in the study. Statistical difference was accepted as $P<0.05$.

2.8. Drugs and Chemicals

All chemicals and drugs unless otherwise stated were obtained from Sigma-Aldrich. Buffer and solution composition are listed in Appendix 1.

CHAPTER 3

THE INFLUENCE OF OESTROGEN AND OESTROGEN RECEPTORS ON THE OUTCOME OF MYOCARDIAL ISCHAEMIA REPERFUSION IN THE FEMALE RAT.

3.1. Introduction

There are two known genomic E receptors that mediate Es' cellular responses. These are discussed fully in Section 1.2.1.1. In the rat myocardium ERα and ERβ are localised to the nuclei of cardiomyocytes fibroblasts and coronary arteries (Grohe *et al.* 1998; Jankowski *et al.* 2001; Nuedling *et al.* 2001; Saunders *et al.* 1997; Yang *et al.* 2004). Similarly, human cardiomyocytes express ERα (Reviewed in Mendelsohn *et al.* 2005) while ERβ is expressed in cardiomyocytes and fibroblasts (Taylor *et al.* 2000).

Experimentally, administration of $17\beta E_2$ reduces the infarct size and inflammatory response after ischaemia-reperfusion (Delyani *et al.* 1996; Hale *et al.* 1996; Squadrito *et al.* 1997), this is discussed in Section 1.6.3. Isolated neutrophils express both ERα and ERβ (Stygar *et al.* 2006) but the receptor mediating the effects of E in reperfusion injury has not been identified.

Experiments using the non-selective ER antagonist ICI 182 780 have demonstrated that the $17\beta E_2$ induced reduction in infarct size is ER mediated, but they do not identify the specific receptor involved (Booth *et al.* 2003; Dubey *et al.* 2001; Hayashi *et al.* 1995). Attempts have been made to investigate the roles of ERα and ERβ in the cardiovascular system using mice genetically altered to remove the ERα. These mouse studies of global ischaemia-reperfusion have reported that the protective properties of E are lost in ERKO mice (Zhai *et al.* 2000). The findings from ERKO mice are limited due to compensatory mechanisms (Karas *et al.* 2001). In this chapter, for investigation of the roles of ERα and ERβ, we used a novel ERα selective agonist, Org37445 and a novel ERβ antagonist Org44488. Dosing regimes were selected on advice from Organon, based on their previous studies.

3.2. Aims

The aim of the current study was to investigate the role of ERα and ERβ in mediating the effects of chronically administered $17\beta E_2$ on infarct size, neutrophil infiltration and oxidative stress following ischaemia-reperfusion in ovx rats. To study the properties of ERα and ERβ we used a novel ERα selective agonist, Org37445 and novel ERβ selective antagonist, Org44488. The preliminary aim of this study was to investigate the sufficient dosage of $17\beta E_2$ to administer to rats after ovx to produce physiological plasma E levels.

3.3. Methods

3.3.1. Dosing of 17β-estradiol

Rats (n=60) underwent ovx as described in Section 2.2.1 and were then randomly assigned to receive either placebo or $17\beta E_2$. In a pilot study, the rats (n=17) assigned to receive $17\beta E_2$ were divided into those receiving 1 or 2 $17\beta E_2$ pellets. This pilot study was designed to find the appropriate number of pellets to implant after ovx in order to achieve the most physiologically relevant $17\beta E_2$ plasma concentration.

3.3.2. Administration of oestrogen receptor selective drugs

Rats underwent ovx as described in Section 2.2.1.1. After ovx the rats were randomly assigned a treatment group as detailed in Table 3.1. The rats were dosed with the novel selective compounds as detailed below and illustrated in Figure 3.1.

Group Treatment
Placebo + Vehicle
E + Vehicle
E + ERβ antagonist
Placebo + ERα agonist
Placebo + ERβ antagonist

Table 3.1. **Hormone and Selective Receptor drug Treatment Groups**

Briefly, the selective ERα agonist has >50 fold selectivity for ERα over ERβ. In an *in vitro* gene transactivation assay (de Gooyer *et al.* 2003), the ERα agonist activated luciferase expression in CHO cells transfected with recombinant human ERα with an EC_{50} of 6.53 x 10^{-11}M. The EC_{50} for activation of human ERβ in the cell system was 5.5 x 10^{-9}M. The ERβ antagonist inhibited gene transactivation induced by $17\beta E_2$ in cells expressing recombinant human ERβ with an IC_{50} of 1.41 x 10^{-8}M and did not inhibit ERα-mediated gene activation up to 10^{-5}M. This illustrates that Org44488 has a greater than 100 fold selectivity for antagonising ERβ compared to ERα.

In vivo the ERα agonist Org37445 or vehicle (5% (w/v) mannitol 0.5% (w/v) gelatin) was administered twice daily (8am and 4pm) for 5 days prior to the experiment by gavage at a dosage of 75 $\mu g.kg^{-1}$. A final dose was administered on the morning of the study. This choice of dose was based on previous observations in an *in vivo* rat anti-osteoporosis assay (Ederveen *et al.* 1999, 2001). In this model Org37445 prevented bone loss and stimulated uterine proliferation, both ERα mediated events. The ERβ antagonist or vehicle (phosphate buffered saline) was administered at a dose of $1mg.kg^{-1}$ for 3 days prior to the experiment by s.c injection (between 10 and 11am). A final dose was given on the morning of the experiment. In previous studies (unpublished) this regime was shown to achieve plasma levels of 300 $nmol.L^{-1}$ of Org44488. Based on *in vitro* data this should be sufficient to fully antagonise the effects of $17\beta E_2$ at ERβ under our study conditions ($0.2nmol.L^{-1}$ plasma oestrogen was achieved by implanted pellets).

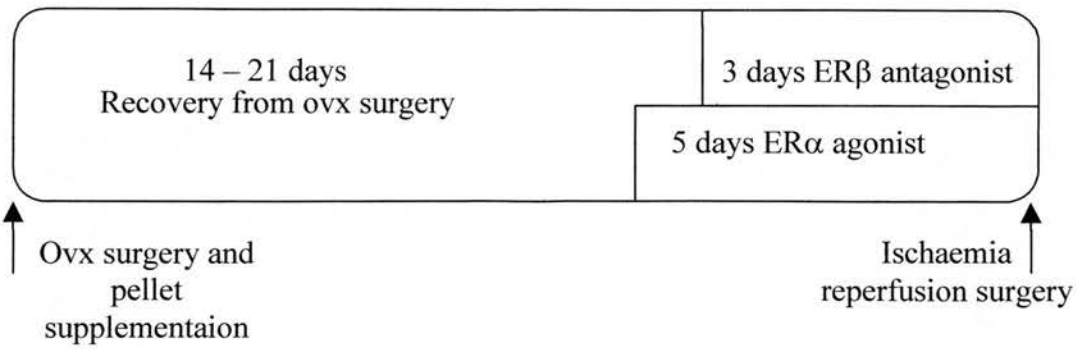


Figure 3.1. ***Timescale of surgery and selective ER drug administration.***

3.3.3. Ischaemia reperfusion

Ischaemia-reperfusion was carried out *in vivo* and *in vitro* as previously described in Section 2.2.2.1 and Section 2.2.2.2, respectively.

3.3.4. Analysis of the Area-at-risk

The AAR was analysed after ischaemia-reperfusion by the modifed Evans blue-TTC technique previously described in Section 2.3.1.

3.3.5. Analysis of neutrophil infiltration

The MPO activity, used as an indication of neutrophil infiltration into the ischaemic region during reperfusion, was assessed as detailed in Section 2.4.1.2.

3.3.6. Measurement of oxidative stress

Oxidative stress within the entire heart and the ischaemic heart after ischaemia-reperfusion was measured by EPR. A detailed description of the procedure used for EPR is in Section 2.6.

3.4. Results

There was a 80% survival rate in this study. There was one rat excluded from the study due to an adverse reaction to the ERα agonist gavage regime, all the other mortalites were during the ischaemia-reperfusion protocol.

3.4.1. Uterine weight and plasma concentration levels

Rats receiving pellets releasing 17βE_2 after ovx had a significantly greater plasma estradiol level compared to ovx rats receiving placebo pellets ($P<0.05$; Figure 3.2a). The rats that were assigned to receive two 17βE_2 releasing pellets had a significantly higher circulating level of plasma estradiol compared to rats that received a single pellet ($P<0.05$; Figure 3.2a).

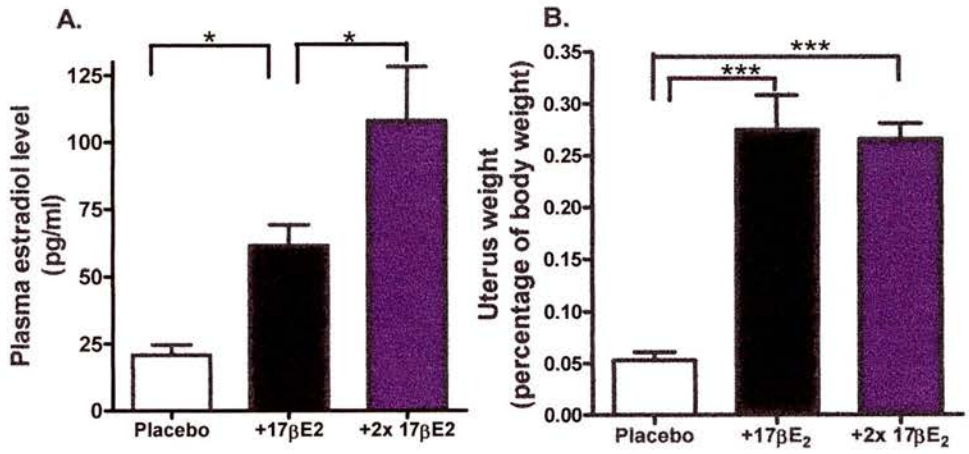


Figure 3.2. **The effect of 17βE_2 pellet supplementation on plasma estradiol and uterine weight.**
*The influence of ovx followed by subcutaneous implantation of placebo and one or two 17βE_2 pellets on (**A.**) plasma estradiol concentration and (**B.**) uterine weight in rats. Data was analysed with a one-way ANOVA followed by a Bonferroni post-hoc test and is expressed as mean ± SEM; n= 5-6; *P<0.05, ***P<0.001.*

Figure 3.2b illustrates that the level of circulating estradiol after ovx had a significant effect on the uterine weight at the time of harvesting. Rats that received one or two 17βE_2 pellets after ovx had a significantly greater uterine weight than rats receiving a

placebo pellet (P<0.001; Figure 3.2b). There was no significant difference between the uterine weight of rats that received one or two $17\beta E_2$ releasing pellets (Figure 3.2b).

3.4.2. *In vivo* ischaemia reperfusion with different doses of $17\beta E_2$

Deprivation of $17\beta E_2$ or the concentration of $17\beta E_2$ replacement did not significantly effect the myocardial AAR that resulted from *in vivo* ischaemia-reperfusion (Figure 3.3).

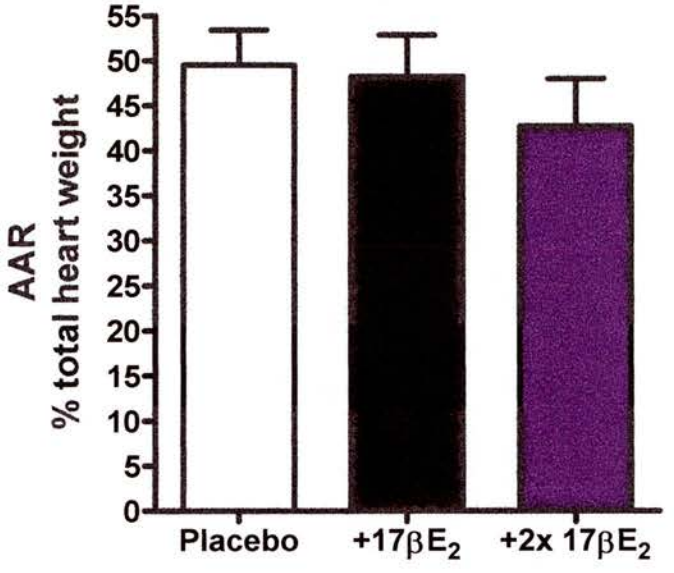


Figure 3.3. **The Area-at-risk after ischaemia-reperfusion in the rat.**
The AAR after in vivo ischaemia-reperfusion in rats with different circulating levels of $17\beta E_2$. The AAR is expressed as a percentage of the total heart weight, these areas were determined with TTC and Evans blue. The data is mean ± SEM; analysed with a one-way ANOVA; n=6, 4 for2x$17\beta E_2$.

The necrotic area within the AAR was significantly decreased in rats treated with $17\beta E_2$ after ovx compared to placebo pellets (Figure 3.4). The reduction in necrotic tissue was more marked with higher circulating levels of $17\beta E_2$ compared to placebo. However, there was no significant difference between the necrotic tissue of rats that were receiving one or two $17\beta E_2$ pellets (Figure 3.4).

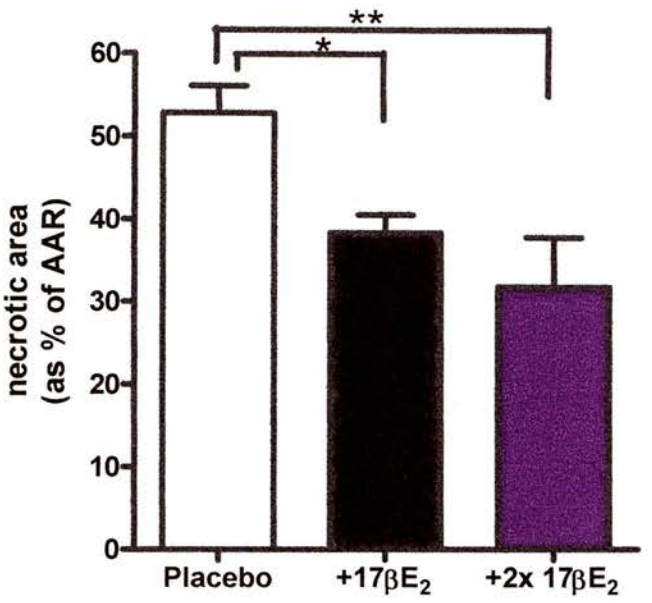


Figure 3.4. **The necrotic myocardial tissue after *in vivo* ischaemia-reperfusion.**
*The necrotic tissue of the myocardium after in vivo ischaemia-reperfusion in the rat expressed as a percentage of the AAR and the effect of varying 17βE$_2$ levels on the amount of necrotic tissue, these areas were determined with TTC and Evans blue. The data was analysed using a one-way ANOVA and a Bonferroni post-hoc test, it is presented as mean ± SEM; n=6, 4 for 2x17βE$_2$; *P<0.05, **P<0.01.*

3.4.3. Body and uterine weight after selective oestrogen receptor agents

All the rats that were assigned to 17βE$_2$ treatment in these studies received a single pellet at the nape of the neck.

The body weight of all the animals was recorded at the time of ovx and at the time of ischaemia-reperfusion. The mean body weight of rats at the time of ovx was 191.6 ± 2.4g. There was no significant difference in body weight at the time of ovx and random assignment to a treatment group. At the time of ischaemia-reperfusion, rats that received 17βE$_2$ or the ERα agonist Org37445 weighed significantly less than placebo/vehicle treated rats (Table 1). Treatment with ERβ antagonist Org44488 did not have any influence on body weight when given to rats receiving placebo or 17βE$_2$ (Table 3.2).

The uterine weight of all animals was recorded at the experimental end point to confirm the effective removal of the ovaries and successful hormone return in the appropriate animals. Animals receiving placebo/vehicle had a significantly lighter uterus ($P<0.05$) than those receiving either $17\beta E_2$ or placebo in combination with selective ERα agonist Org37445 (Table 3.2). The ERβ antagonist Org44488 did not have a significant effect on the uterus weight when given to rats receiving placebo or $17\beta E_2$ (Table 3.2.).

Hormone Treatment	Body weight (BW, g)	Uterine weight/ BW (%)
Placebo + vehicle	278.0 ± 6.4	0.04 ± 0.01
$17\beta E_2$ + vehicle	220.5 ± 6.8***	0.25 ± 0.03***
$17\beta E_2$ + ERβ antagonist	216.6 ± 4.1***	0.23 ± 0.02***
Placebo + ERα agonist	232.6 ± 4.7***	0.12 ± 0.03***
Placebo + ERβ antagonist	279.0 ± 8.9	0.05 ± 0.01

***Table 3.2.* Effect of $17\beta E_2$ and selective ER drugs on the body weight and uterine weight after ischaemia reperfusion in the rat.**
*Data shown is mean ± SEM; Analysed using a one way ANOVA with a Bonferroni post-hoc test, ***P< 0.001 compared to placebo n=6-8.*

3.4.4. *In vivo* ischaemia reperfusion

The MABP was not significantly different between treatment groups before ischaemia (Table 3.3). Throughout ischaemia and reperfusion the MABP did not differ significantly between treatment groups. At the onset of ischaemia there was a drop in MABP, this then recovered during the initial 15 mins of ischaemia to pre-ischaemic pressures and was maintained until the end of the experiment. Treatment with $17\beta E_2$ or selective ER ligands did not have a significant effect on this pattern or on the MABP at the end of the ischaemia and reperfusion periods (Table 3.3).

Treatment	Before ischaemia	Ischaemia onset	Reperfusion	2hrs Reperfusion
Placebo + vehicle	73.1 ± 12.8	43.4 ± 8.5	61.1 ± 14.2	55.4 ± 5.6
17βE$_2$ + vehicle	88.1 ± 4.1	51.2 ± 7.8*	60.0 ± 12.1	62.0 ± 10.3
17βE$_2$ + ERβ antagonist	73.1 ± 13.4	52.3 ± 4.9	56.0 ± 5.4	60.2 ± 4.5
Placebo + ERα agonist	49.6 ± 10.0	36.3 ± 3.0	70.9 ± 10.4	60.3 ± 14.7
Placebo + ERβ antagonist	65.0 ± 11.3	35.1 ± 4.0	65.27 ± 2.1	65.4 ± 3.9

Table 3.3. **The MABP of rats during *in vivo* ischaemia reperfusion.**
*The effect of ovx and selective ER stimulation or ablation on the MABP during the course of ischaemia-reperfusion. The MABP was analysed with a repeated measures ANOVA within groups and a one-way ANOVA at selected time points. Values are mean in mmHg ± SEM; *P<0.05 compared to the treatment group before ischaemis. n =5 or 6.*

The heart rate did not change significantly throughout ischaemia-reperfusion in any treatment group. Different hormonal status only had a significant effect before ischaemia when the heart rate was significantly higher in placebo treated rats with ERβ antagonist Org44488, compared to 17βE$_2$ treated rats with the ERβ antagonist Org44488 (Table 3.4).

Treatment	Before ischaemia	Ischaemia onset	Reperfusion	2hrs Reperfusion
Placebo + vehicle	164.0 ± 11.5	158.0 ± 11.5	166.0 ± 8.4	144.0 ± 7.6
17βE$_2$ + vehicle	152.0 ± 7.4	168.0 ± 7.6	178.0 ± 13.3	180 ± 13.5
17βE$_2$ + ERβ antagonist	146.0 ± 11.8*	156.0 ± 6.9	142.0 ± 6.5	140.0 ± 7.0
Placebo + ERα agonist	146.4 ± 14.9	136.8 ± 9.0	160.8 ± 4.8	159.0 ± 5.7
Placebo + ERβ antagonist	192.0 ± 20.6	178.0 ± 29.8	172.0 ± 7.4	174.0 ± 4.1

Table 3.4. **The heart rate of rats during *in vivo* ischaemia reperfusion.**
*The effect of ovx and selective ER stimulation or ablation on the rat heart rate during the course of ischaemia-reperfusion. The heart rate was analysed using a repeated measures ANOVA within groups and a one-way ANOVA followed by a Bonferroni post-hoc test between groups; *P<0.05 compared to placebo + ERβ antagonist at the same time point. Values are mean ± SEM; n =5 or 6.*

The AAR in the placebo/vehicle treated group was 57.1 ± 3.8 % of the total heart; the AAR in the other treatment groups did not differ significantly from this (Figure 3.5). In the *in vivo* experiments, 17βE$_2$ significantly reduced the size of the necrotic zone within the AAR compared to placebo/vehicle-treated ovariectomised rats (P<0.05; Figure 3.6). Treatment with the ERβ antagonist Org44488 did not significantly alter this 17βE$_2$ induced decrease in necrotic tissue (Figure 3.6a). Treatment with the ERβ antagonist Org44488 after placebo treatment had no significant effect compared to vehicle (Figure 3.6a). Placebo-treated rats receiving the ERα agonist Org37445 also displayed a significant reduction in necrotic tissue compared to placebo/vehicle-treated rats (P<0.01; Figure 3.6b).

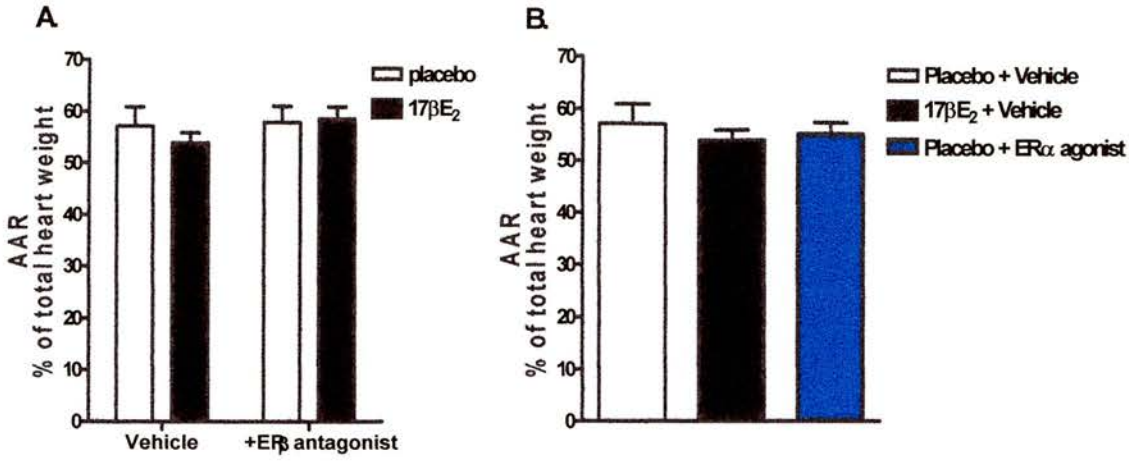


Figure 3.5. **The Area-at-risk after *in vivo* ischaemia reperfusion.**
*The area of myocardium at risk after in vivo ischaemia-reperfusion in the rat and the influence17βE_2 with or without selective (**A.**) ERβ antagonist Org44488 and (**B.**) ERα agonist Org37445 on this, expressed as a percentage of the total heart weight, these areas were determined with TTC and Evans blue. The data was analysed using a (**A.**) two-way ANOVA and (**B.**) a one-way ANOVA both followed by a Bonferroni post-hoc test, it is expressed as the mean ± SEM; n=6, 8 for placebo + ERβ antagonist.*

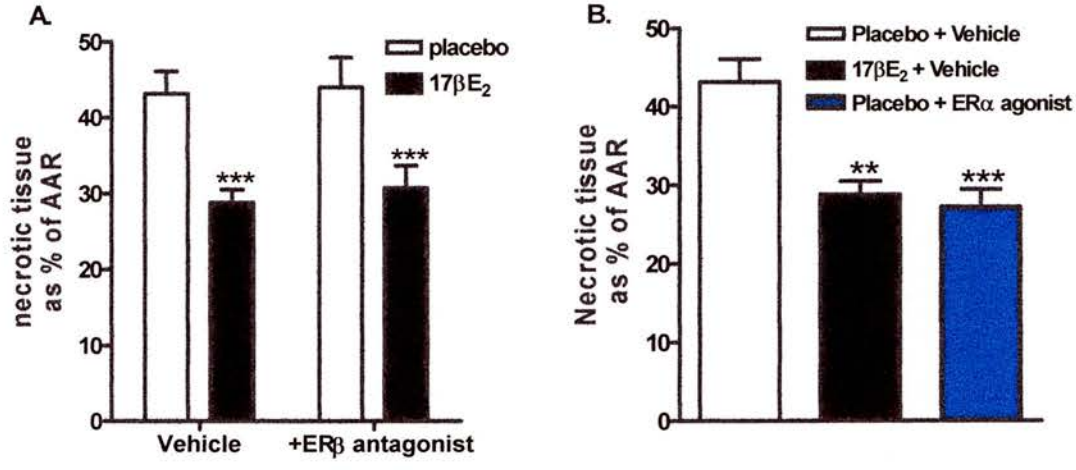


Figure 3.6. **The necrotic myocardium after *in vivo* ischaemia reperfusion.**
*The necrotic myocardium of rats having undergone in vivo ischaemia-reperfusion after ovx and chronic treatment with 17βE_2 and selective (**A.**) ERβ antagonist Org44488 or (**B.**) ERα agonist Org37445. The necrotic myocardium is expressed as a percentage of the AAR, these areas were determined with TTC and Evans blue. The data is presented as the mean ± SEM. The data was analysed using a (**A.**) two-way ANOVA and (**B.**) a one-way ANOVA both followed by a Bonferroni post-hoc test; **P<0.01; ***P<0.001 compared to placebo treatment; n=6, 8 for placebo + ERβ antagonist.*

3.4.5. *In vivo* neutrophil infiltration

Tissue MPO activity was measured and used as a marker of neutrophil infiltration following reperfusion. Supplementation with $17\beta E_2$ after ovx significantly ($P<0.05$) reduced the amount of MPO activity detected in the AAR compared to that in hearts from placebo/vehicle-treated rats (Figure 3.7). Dosing placebo-treated rats with the selective ERα agonist Org37445 also significantly reduced the level of MPO activity within the AAR, reproducing the effect of $17\beta E_2$ ($P<0.05$; Figure 3.7).

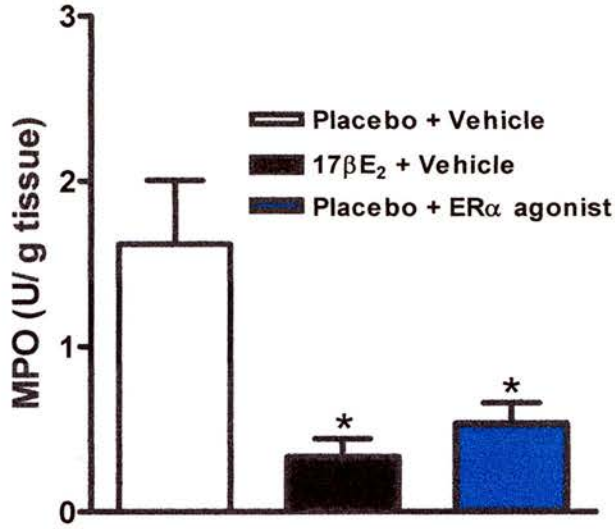


Figure 3.7. **Myeloperoxidase activity within the area-at-risk after ischaemia reperfusion.**
*The MPO activity in the myocardial AAR after in vivo ischaemia-reperfusion in the rat and the influence of ovx and $17\beta E_2$ or selective ERα stimulation on the activity. The activity was measured at 405nm and is expressed as MPO activity per gram of myocardial AAR. The data was analysed using a one-way ANOVA and a Bonferroni post-hoc test, it is expressed as the mean ± SEM; *$P<0.05$; n=5, 6 for the placebo treatment.*

3.4.6. Free radical production after *in vivo* ischaemia reperfusion

Neutrophils are a major source of oxidative stress following reperfusion. Therefore any change in neutrophil infiltration should be correlated with a reduction in free radical production from the AAR. EPR was used to detect the influence of $17\beta E_2$, ERα agonist and ERβ antagonist treatment on free radical production by the reperfused AAR of the myocardium following *in vivo* ischaemia-reperfusion. In hearts from placebo/vehicle treated animals significantly more free radicals were detected in the perfusate collected from the whole heart than in perfusate from the non-ischaemic myocardium alone, demonstrating free radical production from the AAR (Figure 3.8; $P<0.01$).

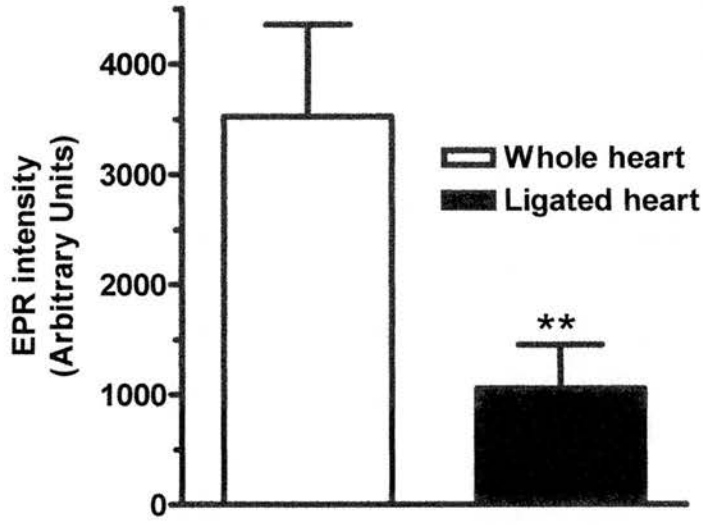


Figure 3.8. **The EPR signal from rat myocardium after *in vivo* ischaemia reperfusion.**
*The EPR signal gained from the perfusate of the entire myocardium and from the myocardium not at risk of infarction (ligated heart), after in vivo ischaemia-reperfusion. The data was analysed using a paired two-tailed t-test and is expressed as the mean ± SEM; **P<0.01; n=5.*

The majority of the oxidant stress could be accounted for by superoxide radicals, as administration of SOD along with the spin trap significantly decreased free radical production from the reperfused heart by 53 ± 10.26% (P=0.04; Figure 3.9).

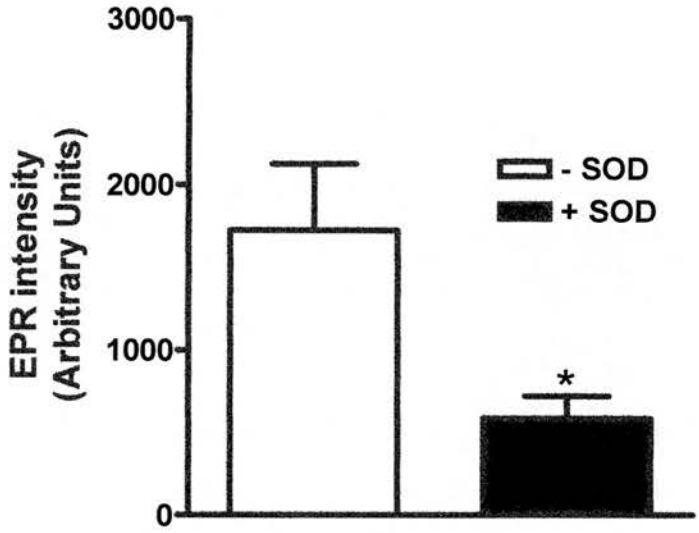


Figure 3.9. **The EPR signal with and without SOD, from rat myocardium after *in vivo* ischaemia reperfusion.**
*The EPR signal recorded from the perfusate of in vivo reperfused infarcted rat myocardium in the presence and absence of SOD. The data was analysed using a paired two-tailed t-test; *P<0.05; the data is presented as the mean ± SEM; n=7.*

Significantly less free radicals were generated from the AAR of hearts from animals that received $17\beta E_2$ compared to those that received placebo/vehicle following ovx ($P<0.01$; Figure 3.10). This influence of $17\beta E_2$ was not modified in hearts from ERβ receptor antagonist treated rats ($P<0.01$; Figure 3.10). Rats that received placebo and the ERα agonist Org37445 also had significantly ($P<0.01$; Figure 3.10.) decreased free radical production compared to the rats treated with placebo/vehicle.

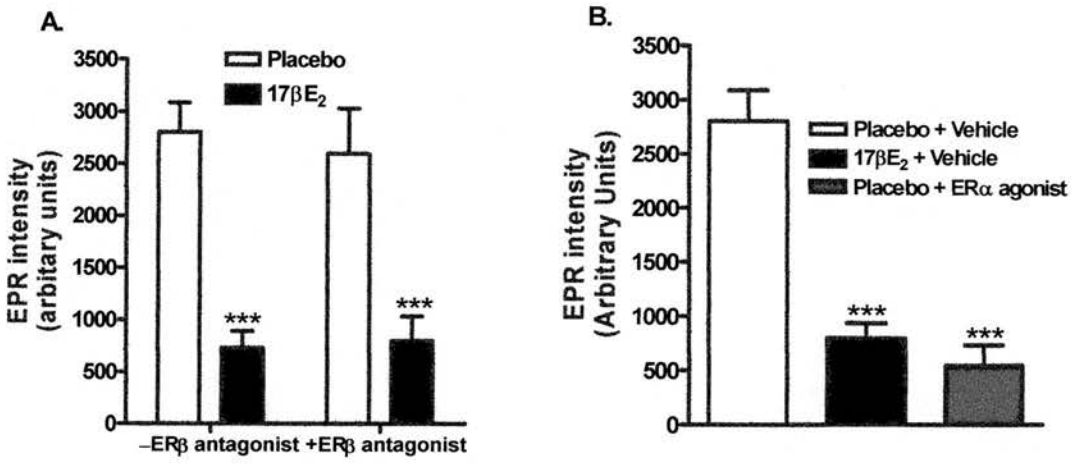


Figure 3.10. **The EPR signal from the AAR of rat myocardium exposed to various ER stimulation prior to *in vivo* ischaemia reperfusion.**
*The mean ± SEM EPR signal from the AAR of rat myocardium after in vivo ischaemia-reperfusion and the effect of chronic ovx and (**A.**) 17βE$_2$ treatment along with selective ERβ antagonist Org44488 (**B.**) or ERα agonist Org37445 treatment on the EPR signal. The data was analysed using a (**A.**) two-way ANOVA and (**B.**) a one-way ANOVA both followed by a Bonferroni post-hoc test; ***P<0.001 compared to placebo/vehicle treatment, n=5-8.*

3.4.7. *In vitro* ischaemia reperfusion

To determine whether selective activation of the ERα could also mimic the direct, neutrophil independent cardioprotective influence of 17βE$_2$ on the myocardium further experiments were conducted in buffer-perfused hearts. The perfusion pressure in hearts from placebo/vehicle-treated rats was not significantly different from placebo rats chronically treated with ERα Org37445 treated rats (Table 3.5). The perfusion pressure dropped at the onset of ischaemia but recovered during the first 15mins of ischaemia and was then maintained at this level until the end of reperfusion.

Treatment	Before ischaemia	Ischaemia onset	Reperfusion	2hrs Reperfusion
Placebo/Vehicle	48.6 ± 3.1	27.2 ± 2.8*	30.44 ± 2.8	46.5 ± 6.5
Placebo/ERα agonist	45.0 ± 10.7	33.8 ± 6.8	45.6 ± 7.4	53.1 ± 8.1

Table 3.5. Mean perfusion pressure of rat myocardium during *in vitro* ischaemia reperfusion.

*The mean perfusion pressure of rat myocardium subjected to in vitro ischaemia-reperfusion and the effect of ovx and chronic 17βE$_2$ or selective ERα agonist Org37445 treatment on this. The data was analysed using a repeated measures ANOVA with a Bonferroni post test within groups and a two-tailed unpaired t-test between groups; *P<0.05 compared to placebo/vehicle before ischaemia; n=6. The data is presented as mean in mmHg ± SEM.*

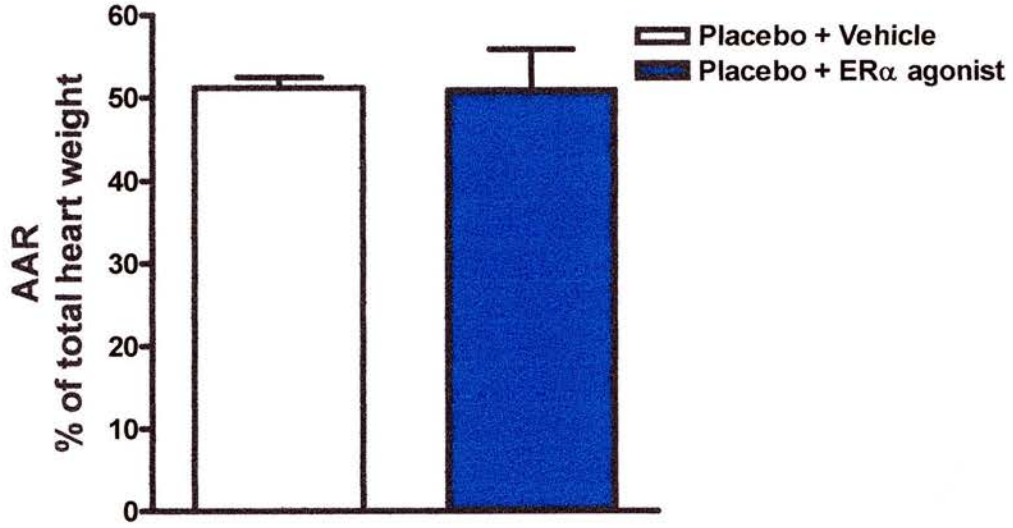


***Figure 3.11.* The area-at-risk in the rat myocardium after *in vitro* ischaemia reperfusion.**

The influence of ovx and chronic treatment with selective ERα agonist Org37445 on the AAR in the rat myocardium after ischaemia-reperfusion in an in vitro neutrophil free perfused set up. The AAR is expressed as a percentage of the total heart weight, these areas were determined with TTC and Evans blue. The data was analysed using a two-tailed unpaired t-test and is expressed as mean ± SEM; n=6.

ERα agonist Org37445 treatment after ovx had no significant effect on the total AAR compared to the placebo/vehicle treated group after *in vitro* ischaemia-reperfusion (Figure 3.11). The AAR was also comparable to that seen *in vivo*. The necrotic area within the AAR was significantly reduced in hearts taken from animals treated with selective ERα agonist Org37445 compared to placebo/vehicle ($P<0.01$; Figure 3.12).

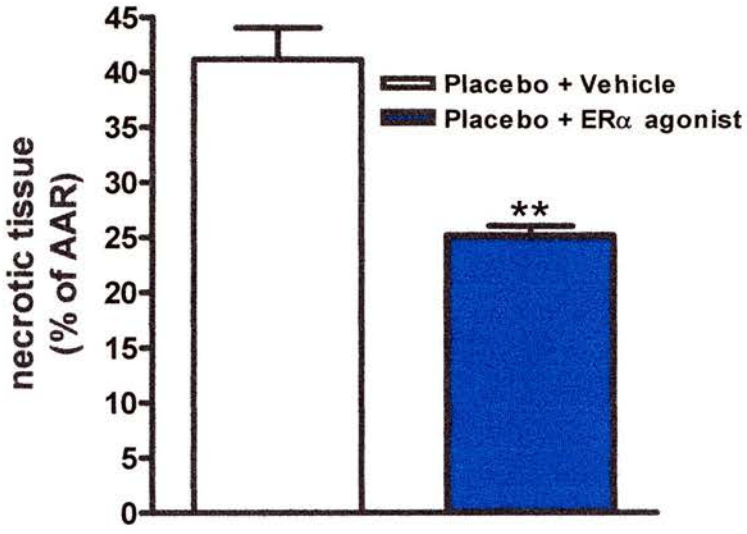


***Figure 3.12.* The necrotic myocardium of rats after *in vitro* ischaemia reperfusion.**
*The mean ± SEM of the necrotic myocardium of rats that have undergone in vitro ischaemia-reperfusion after ovx and chronic treatment with selective ERα agonist Org37445 compared to complete deprivation of 17βE_2. The necrotic myocardium is expressed as a percentage of the AAR, these areas were determined with TTC and evans blue. The data was analysed using a two-tailed unpaired t-test; **$P<0.01$; $n=6$.*

3.5. Discussion

At the time of this study the role of ERα and ERβ during chronic administration of $17\beta E_2$ in an experimental model of ischaemia-reperfusion was not known. Therefore, the aim of this study was to identify the roles of ERα and ERβ in mediating the cardioprotection provided by chronic administration of $17\beta E_2$ in a model of ischaemia-reperfusion. This is the first study to use a selective ERβ antagonist to investigate the role of ERs in ischaemia-reperfusion. Previous studies in mice have failed to provide a clear answer as the protective effects of E in ischaemia-reperfusion are reported to be lost in both ERKO (Wang *et al.* 2006) and BERKO mice (Gabel *et al.* 2005). Consequently, we employed novel ER selective agonists and antagonists in the rat to investigate the role of individual receptors. The main finding of this chapter was that chronic administration of the selective ERβ antagonist Org44488 with $17\beta E_2$ did not attenuate the $17\beta E_2$ associated decrease in infarct size, neutrophil infiltration and oxidative stress after ischaemia-reperfusion. We also found that chronic administration of the selective ERα agonist Org37445 to placebo treated rats reproduced the $17\beta E_2$ mediated decrease in infarct size, neutrophil infiltration and oxidative stress. These results suggest that ERα is the predominant ER involved in cardioprotection by $17\beta E_2$.

In order to study the role of the individual ERs during ischaemia-reperfusion the ovaries which are the main natural source of E were removed and the rats were implanted with pellets releasing a controlled level of $17\beta E_2$ or placebo. In the preliminary study, we determined that implanting a single pellet releasing 0.05 $mg.day^{-1}$ of $17\beta E_2$ was sufficient to obtain a plasma E level significantly greater than placebo treated rats and in the mid-range of physiological levels. In comparison the rats that received two $17\beta E_2$ pellets, and therefore double the daily $17\beta E_2$ had a plasma E concentration that was equivalent to the high proestrus level (100-150$pg.ml^{-1}$). The pilot study also confirmed that the low dose of $17\beta E_2$ had the same cardioprotective properties as the high dose, they both failed to have a significant effect on the AAR but significantly decreased the

necrotic area of the myocardium after ischaemia-reperfusion compared to the placebo treated rats. We deemed that constant plasma E levels in the mid cycle range was of greater physiological relevance than the higher levels. The result that the cardioprotection provided by both doses of $17\beta E_2$ was comparable ensured the lower dose of $17\beta E_2$ was suitable for the continuing study.

The model of ischaemia used causes damage to the myocardium due to hypoxia-induced vasoconstriction and tissue necrosis (Jennings *et al.* 1983; Lefer 1987), as well as reperfusion associated infiltration of activated inflammatory cells, increased oxidative stress, calcium overload and reduced tissue ATP levels (Hudson 1994; McCord 1985; Premaratne *et al.* 1993). It is well established that $17\beta E_2$ provides protection in experimental models of ischaemia-reperfusion (Mendelsohn *et al.* 1999; Stumpf *et al.* 1977). Several mechanisms have been proposed to account for the protective effects of E in this model (Levine *et al.* 1996; Lucchesi 1990; Meade *et al.* 1992; Stampfer *et al.* 1991; Sullivan *et al.* 1995; Wahl *et al.* 1983). Among these, stimulation of NO synthesis and activity may play a central role (Rubanyi *et al.* 1997). Increased NO bioavailability not only leads to increased vasodilation and reduction of hypoxia, but also contributes to inhibition of inflammatory cell infiltration (Davies *et al.* 1995). As well as stimulated NO bioavailability, E mediated protection is partly through inhibition of neutrophil infiltration (Squadrito *et al.* 1997) and a reduction of oxidative damage to the myocardium (Bush *et al.* 1987; Squadrito *et al.* 1997; Sugioka *et al.* 1987). This protection is likely to involve attenuated pro-apoptotic JNK and p38 MAPK activity and a simultaneous increase in anti-apoptotic ERK activity (Vilatoba *et al.* 2005; Wang *et al.* 2006), that lead to a reduced necrotic zone. Regulation of these pathways is likely to be through non-genomic pathways and regulated through G-protein coupled receptors. In the present study we confirmed that both infarct size and neutrophil infiltration, assessed by myocardial myeloperoxidase activity, were reduced in treated animals.

The current results allow us to conclude that the cardioprotection displayed by $17\beta E_2$ and ERα agonist Org37445 in this study is through both neutrophil dependent and

independent mechanisms. The neutrophil dependent mechanisms that we can allude to being important in this study are reduced neutrophil infiltration and consequently oxidative stress.

Oxidative stress is an important cause of injury following tissue reperfusion. Previous studies have used assays to detect the end products of oxidative damage (Barp *et al.* 2002; Persky *et al.* 2000). These experiments have concluded that treatment with $17\beta E_2$ decreases the oxidative stress and increases the level of SOD activity (Barp *et al.* 2002). These methods of detecting free radical production and oxidative damage are not very accurate because they record the resulting product. Detecting the free radicals directly provides a better indication of their production. Therefore, in the present study we additionally aimed to measure free radical release from the reperfused myocardium using EPR. The methods of EPR with the advantages of it are discussed in Section 2.6.

This is the first time that EPR has been used to assess free radical production from hearts that have undergone *in vivo* ischaemia-reperfusion. *In vitro* perfusion of hearts with the CP-H spin trap after *in vivo* ischaemia-reperfusion was successful in detecting free radical release. Free radical detection was significantly reduced when perfusion through the AAR was prevented, identifying the AAR as the major site of free radical production in these hearts. The most likely source of oxidative stress within the AAR is neutrophils that have infiltrated during reperfusion (McCord 1985). In the present study the reduction in EPR signal after dual perfusion of spin-trap and SOD demonstrates that the majority of free radicals produced in the myocardium are super oxide anions, the predominant free radical released from neutrophils (Tauber *et al.* 1977). Treatment with $17\beta E_2$ clearly reduced the oxidant signal generated in the myocardium after ischaemia-reperfusion compared to placebo/vehicle treated rats. This reduction corresponds to the reduction in neutrophil infiltration, seen by attenuated MPO activity in $17\beta E_2$ treated compared to placebo/vehicle treated animals. In the reperfused heart, damaged mitochondria are also a source of oxidative stress, through disruption of the electron transfer chain (Lesnefsky *et al.* 2001) and may contribute here. $17\beta E_2$ has previously

been shown to reduce the oxidative stress from isolated rat mitochondria (Stirone *et al.* 2005) and may be an additional mechanism of inhibited oxidative stress in the current model of damage. $17\beta E_2$ also up-regulates endogenous antioxidant systems, such as glutathione peroxidase and SOD through enhanced MAPK and NF_KB activity (Borras *et al.* 2005). The pathways involved here are likely to involve a non-genomic ER. Thus free radical scavenging may also be enhanced following chronic treatment with $17\beta E_2$.

Cardioprotection of $17\beta E_2$ and ERα activation in particular, through a neutrophil independent mechanism acting within the myocardium can be alluded to due to the *in vitro* results in this study. Neutrophil independent mechanisms may involve the action of fibroblasts within the myocardium as these are known to express ERs (Lee *et al.* 1998; Mahmoodzadeh *et al.* 2006).

The principal aim of this study was to identify the roles of ERα and ERβ in mediating the cardioprotective effects of E. ERα is responsible for the stimulation of epithelial cell proliferation in the uterus that results in the observed increase in uterine weight of rats treated with $17\beta E_2$ or ERα agonist (Frasor *et al.* 2003). ERα also mediates the $17\beta E_2$ induced attenuation of food intake and weight gain (Roesch 2006). Administration of the ERα agonist Org37445 increased uterine and decreased body weight, consistent with successful activation of this receptor. The ERβ antagonist Org44488 had no significant effect on the uterine or body weight of rats treated with $17\beta E_2$ consistent with a lack of blockade of ERα.

The ERβ antagonist Org44488 did not show any significant effects when administered with placebo. The ERβ antagonist Org44488 did not attenuate the effects of $17\beta E_2$ on infarct size, or the decrease in free radical detection after treatment with $17\beta E_2$, which suggests that it is ERα that mediates the attenuated infarct size and free radical production in the myocardium after ischaemia-reperfusion. ERβ has been detected in the rat heart (Grohe *et al.* 1998; Jankowski *et al.* 2001; Yang *et al.* 2004), and it has been

implicated in mediating cardioprotection following trauma-haemmorhage (Yu *et al.* 2006). ERβ is also highly expressed in the neonatal and developing heart when it is under going development, after which the expression decreases (Jankowski *et al.* 2001). However, it does not appear to have a role in mediating cardioprotection by E following ischaemia-reperfusion. The results of the present study suggest that $17\beta E_2$ is more likely to act through stimulation of ERα in the rat heart. ERα expression is low in the neonatal rat heart and increases to a relatively high level in the adult heart (Jankowski *et al.* 2001). Thus suggesting that ERα modulates the mature myocardium and acute effects, such as those we detected in this study. This conclusion is supported by the fact that the ERα agonist Org37445 reproduced the *in vivo* and *in vitro* cardioprotective effects of $17\beta E_2$, including attenuated neutrophil infiltration, oxidative stress and cell necrosis, all the parameters that were measured in the current study.

In another recent study, acute administration of the selective ERα agonist (4,4,4-4 propyl-(1H)-pyrazole 1,3,5-triyltrisphenol-PPT) PPT 30 minutes before ischaemia and reperfusion reduced infarct size in the rabbit, while the ERβ agonist DPN (2,3-bis(4 hydroxyphenyl propionitrole-DPN) failed to provide any cardioprotection (Booth *et al.* 2005). In this study, cardioprotection via stimulation of ERα was linked to a reduction of C-reactive protein (CRP) deposition within the infarct area. Recently a study has demonstrated that CRP can enhance infarct damage following permanent ligation of the coronary artery in the rat (Pepys *et al.* 2006). Reduction of CRP deposition is also likely to be a feature of protection following chronic stimulation of ERα in the present study. CRP is thought to be a predictor of cardiovascular events (Khreiss *et al.* 2004), I believe that caution should be maintained regarding the use of CRP levels as an indication of protection or detriment to the infarct. Plasma levels of CRP are elevated upon a host of injuries and are therefore difficult to specifically relate to myocardial injury (Yaron *et al.* 2006. A further note for caution is highlighted by the observation that weight loss such as that seen in 17(E2 treated rats is known to reduce plasma CRP{Selvin, 2007 #137; Zouki *et al.* 2001). Therefore, additional mechanisms will also contribute e.g. enhanced

expression of endothelial nitric oxide synthase and reduced expression of adhesion molecules, effects of E that reduce neutrophil infiltration (Delyani *et al.* 1996; Squadrito *et al.* 1997), and have previously been linked to activation of ERα (Schrepfer *et al.* 2006; Tan *et al.* 1999).

Stirone *et al* have recently reported that activation of ERα in isolated mitochondria from cerebral arteries inhibits oxidative stress, and that this property of ERα is abolished in the presence of ICI-182 780 (Stirone *et al.* 2005), this is a further potential mechanism through which ERα activation may attenuate oxidative damage in the myocardium. The results of the *in vitro* study show that there is also neutrophil independent cardioprotection following chronic stimulation of ERα.

The current study is the first study to demonstrate, using a novel combination of ERα agonist and ERβ antagonist, that the cardioprotective properties of $17\beta E_2$ in a rat model of ischaemia-reperfusion are mediated via stimulation of the ERα.

CHAPTER 4

MEDROXYPROGESTERONE ACETATE INHIBITS THE CARDIOPROTECTIVE EFFECT OF OESTROGEN IN EXPERIMENTAL ISCHAEMIA REPERFUSION INJURY.

4.1. Introduction

Experimental models of CVD and injury support the concept that E has protective properties within the cardiovascular system. Within rat and mouse models of ischaemia-reperfusion E is known to reduce myocardial injury, due at least in part to its ability to reduce neutrophil infiltration into the ischaemic myocardium (Kolodgie *et al.* 1997; Squadrito *et al.* 1997; Zhai *et al.* 2000). Prior to the results of the double blinded randomised clinical trials in 2002 (Hulley *et al.* 1998; Rossouw *et al.* 2002) there had been limited research on the effect of progestogens on the cardioprotective properties of E. Progestogens are added to ERT to circumvent detrimental cell proliferation in the uterus, which arises from uninhibited E treatment in women having undergone a hysterectomy. Progestogens have their own biological effects, the limited studies prior to commencing this one provided evidence of them acting synergistically with E, having no influence and or antagonising Es' effects (Hanke *et al.* 1996; Simoncini *et al.* 2003). MPA has frequently been used in the clinical trials carried out to date and has proved particularly problematic. Several experimental and clinical studies have shown that MPA can negate the positive effects of E on endothelial function (Wakatsuki *et al.* 2001) and its ability to attenuate neointima formation following vascular injury (Levine *et al.* 1996).

4.2. Aim

The aim of the present study was to investigate the influence of MPA on the proven protective effect of $17\beta E_2$ in an experimental model of ischaemia-reperfusion injury. We hypothesize that co-administration of MPA with $17\beta E_2$ attenuates the cardioprotective properties of $17\beta E_2$ during ischaemia-reperfusion in the rat. Hearts from ovx rats chronically treated with hormones were studied both *in vivo* and *in vitro,* to determine the relative importance of systemic and local interactions.

4.3. Methods

4.3.1. Silicon pellets

In house pellets were used during this study. The pellets were made using silicon tubing (2.4mm diameter), cut to 2-3mm in length. NuSil RTV silicon adhesive was used to seal one end prior to filling the tubing with $17\beta E_2$ or MPA. 3mg of $17\beta E_2$ or MPA was used per pellet, once filled the second end of the tubing was sealed with NuSil RTV silicon adhesive. Placebo pellets were left empty. Once the adhesive had dried the filled and sealed pellets were stored in sterile saline at $4^{O}C$. These in-house pellets have been used previously in experimental models investigating the effect of $17\beta E_2$, and were demonstrated to achieve a plasma level of E within physiological limits (less than 500 pg/ml; Fink *et al.* 1998)

4.3.2. Hormone Administration

Female rats (n=61) were prepared for surgery as described in Section 2.2 and then under went ovx as described in Section 2.2.1.1. Rats that were randomly assigned to MPA or $17\beta E_2$ hormone treatment received a pellet containing MPA or $17\beta E_2$ respectively. Rats assigned to the combined hormone treatment of $17\beta E_2$ and MPA were implanted with 2 separate pellets, one containing $17\beta E_2$ and one with MPA.

4.3.3. Experimental Ischaemia reperfusion

In vivo and *in vitro* ischaemia-reperfusion was induced at least 14 days after ovx surgery and pellet supplementation, as described in Section 2.2.2.1 and 2.2.2.2 respectively.

4.3.4. Analysis of myocardial tissue

The myocardial AAR was determined and analysed after *in vivo* and *in vitro* ischaemia-reperfusion as described in detail in Section 2.3.1. Neutrophil infiltration in the AAR was also measured after *in vivo* ischaemia-reperfusion through an MPO activity assay, this protocol is described in detail in Section 2.4.1.

4.4. Results

This study had a 77% survival rate. All the mortalities were during the ischaemia-reperfusion period, there were not any mortalities during ovariectomy.

4.4.1. Body and Uterine weight

There was no significant difference between the body weights of rats in the different treatment groups at the beginning of the study (Table 4.1). At the end of the study, ovx rats treated with $17\beta E_2$ weighed significantly less than animals receiving placebo or MPA alone ($P<0.01$, Table 4.1.). Co-administration of MPA failed to influence this effect of $17\beta E_2$. Treatment with $17\beta E_2$ also resulted in an increase in uterine weight relative to placebo treatment ($P<0.001$, Table 4.1.). MPA had no effect on uterine weight when given alone and had no influence on the ability of E to increase the uterine weight (Table 4.1).

Treatment	Initial Body Weight (g)	Final Body Weight (g)	Uterine Weight (% of final body weight)
Placebo	199.6 ± 6.7	272.8 ± 9.4	0.092 ± 0.01
$17\beta E_2$	199.5 ± 5.2	231.0 ± 8.4**	0.262 ± 0.02**
MPA	191.6 ± 8.5	254.4 ± 11.6	0.120 ± 0.01
$17\beta E_2$ + MPA	202.7 ± 6.7	229.8 ± 4.4**	0.272 ± 0.02**

Table 4.1. **Body and uterine weights of the rat, the effect of $17\beta E_2$ and MPA.**
*Pre-surgery and final body weight of rats used in vivo, in vitro and for the MPO study. Final body weights are a minimum of 14 days post ovariectomy and hormone supplementation. The uteri were weighed up on removal from the animal at the end of the experiment. The uteri were dried in tissue prior to being weighed. Values shown are mean ± S.E.M, the data was analysed using a one-way ANOVA with a Bonferroni post hoc test was used, **$P<0.01$; $n= 14-19$.*

4.4.2. *In vivo* ischaemia reperfusion

No treatment significantly altered the size of the AAR (Figure 4.1.). Despite there being no difference in the overall AAR, treatment with $17\beta E_2$ alone significantly reduced the necrotic zone within it, compared to placebo treatment ($P<0.05$, Figure 4.2.). Treatment with MPA and $17\beta E_2$ in combination resulted in a significantly ($P<0.05$) larger necrotic zone compared to that in animals treated with $17\beta E_2$ alone, such that there was no longer protection relative to placebo treatment (P= NS compared to placebo, Figure 4.2.).

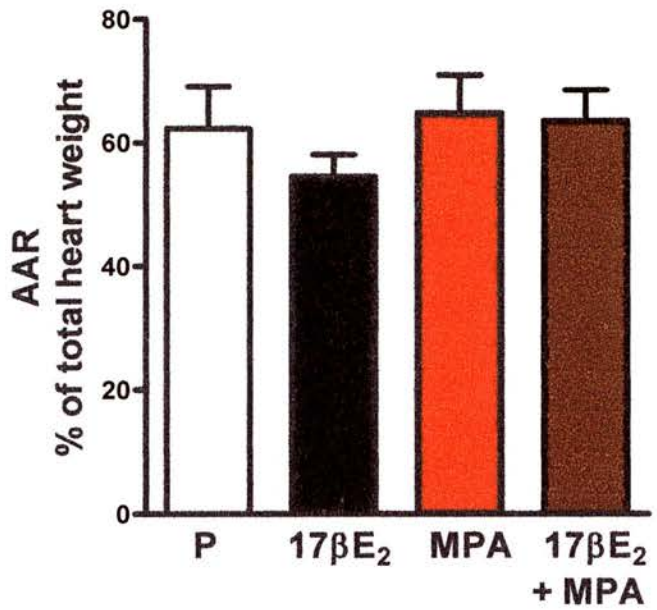


Figure 4.1. **The AAR after *in vivo* ischaemia-reperfusion in the rat.**
The AAR after in vivo ischaemia-reperfusion in the rat, it is expressed as a percentage of the total heart weight. Data was analysed using a One-way ANOVA; data shown is mean ± SEM; n= 4-5.

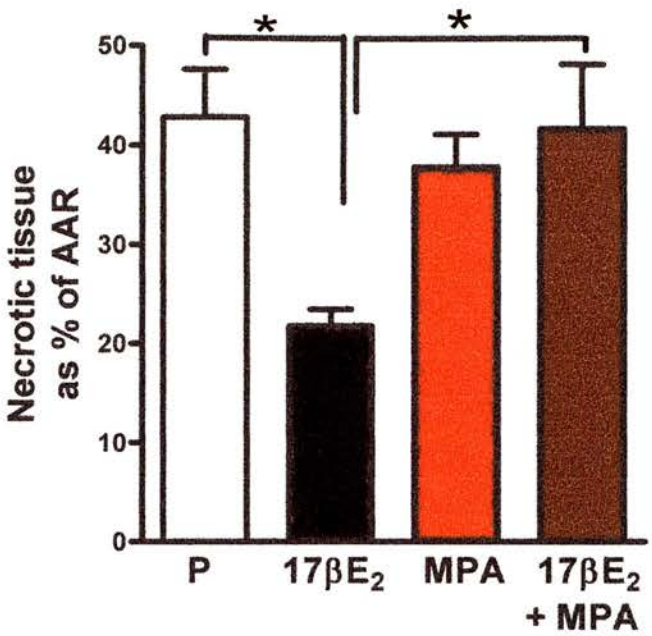


Figure 4.2. **Necrotic myocardium after *in vivo* ischaemia-reperfusion.**
*The necrotic area of the myocardium, expressed as a percentage of the AAR after in vivo ischaemia-reperfusion in the rat. Data was analysed using a one-way ANOVA with a Bonferroni post-hoc test; data shown is mean ± SEM; *P<0.05; n=4-5.*

The MABP before ischaemia in the placebo treated group was 71.8 ± 4.0 mmHg, this was not significantly different between treatment groups (Table 4.2). There was a significant ($P<0.05$) drop in pressure at the onset of ischaemia, the MABP recovered during the first 30 mins of ischaemia to values not significantly different to before the onset of ischaemia. The MABP remained constant throughout reperfusion but dropped off at the end of reperfusion (Table 4.2). None of the treatments influenced the characteristic change in blood pressure following ischaemia or reperfusion (Table 4.2). The heart rate remained relatively constant throughout ischaemia-reperfusion and was not significantly different between treatment groups (Table 4.3).

Treatment	Before ischaemia	Ischaemia onset	Reperfusion	2hrs Reperfusion
Placebo	71.3 ± 7.8	40.9 ± 4.0*	72.5 ± 11.5	37.2 ± 5.0**
17βE$_2$	76.9 ± 9.3	42.4 ± 5.5**	65.9 ± 7.8	48.0 ± 5.8*
MPA	72.9 ± 8.2	44.8 ± 5.4*	62.6 ± 8.2	43.3 ± 6.3*
17βE$_2$ + MPA	67.0 ± 6.4	35.9 ± 5.8*	52.0 ± 9.3	37.5 ± 11.7*

Table 4.2. **MABP during *in vivo* ischaemia reperfusion.**
*The mean arterial blood pressure (MABP) in mmHg throughout ischaemia-reperfusion in the rat. The data was analysed using a repeated measures ANOVA within groups and a one-way ANOVA between groups at each time point. The ANOVA was followed by a Bonferroni post-hoc test, the values shown are mean ± SEM; **P<0.01 *P<0.05; n= 4-6.*

Treatment	Before ischaemia	Ischaemia onset	Reperfusion	2hrs Reperfusion
Placebo	361.8 ± 3.8	365.4 ± 34.3	393.6 ± 39.4	357.0 ± 32.3
17βE$_2$	407.8 ± 41.2	394.8 ± 36.2	426.0 ± 48.6	338.7 ± 30.8
MPA	327.8 ± 36.3	334.5 ± 24.6	335.2 ± 28.8	297.6 ± 32.2
17βE$_2$ + MPA	339.3 ± 36.8	323.0 ± 36.3	360.0 ± 37.6	383.0 ± 30.1

Table 4.3. **The heart rate during *in vivo* ischaemia reperfusion.**
The mean heart rate during ischaemia-reperfusion in the rat. The data was analysed using a repeated measures ANOVA within each group and a one-way ANOVA between groups at each time point. Data shown is mean ± SEM; n= 4-6.

4.4.3. Myeloperoxidase activity

To further investigate the effect of MPA on the protective properties of $17\beta E_2$, the MPO activity within the myocardial AAR after *in vivo* ischaemia-reperfusion was determined. MPO activity is a good indication of the neutrophil infiltration into the area. Supplementation with $17\beta E_2$ significantly reduced the amount of MPO activity in the AAR compared to placebo supplementation ($P<0.05$; Figure 4.3.). MPA supplementation alone did not significantly alter the MPO activity compared to placebo treatment. However, when MPA and $17\beta E_2$ were supplemented in combination the amount of MPO activity in the AAR was significantly increased compared to treatment with $17\beta E_2$ alone ($P<0.05$, Figure 4.3.).

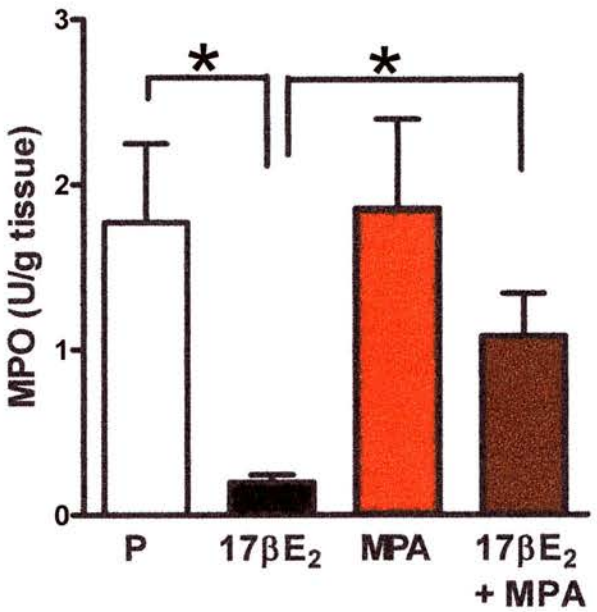


Figure 4.3. **Myeloperoxidase (MPO) activity in the AAR.**
*MPO activity in the AAR of the myocardium after in vivo ischaemia-reperfusion in the rat. Data was analysed using a one-way ANOVA and Bonferroni post-hoc test; data shown is mean ± SEM; *P<0.05; n=6-4.*

4.4.4. *In vitro* ischaemia reperfusion

A similar pattern was seen as *in vivo*. The size of the AAR *in vitro* was comparable to that *in vivo* and again there was no significant difference between the AAR in any treatment group (Figure 4.4).

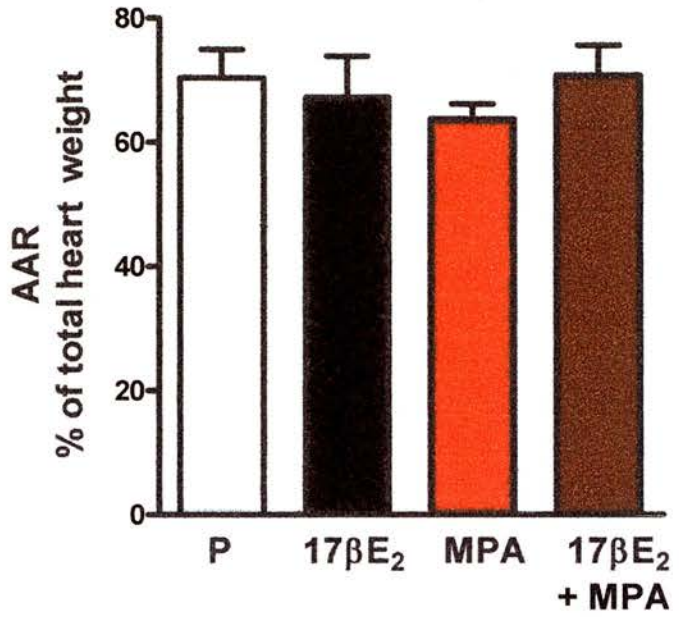


Figure 4.4. **The AAR after *in vitro* ischaemia reperfusion.**
The myocardial AAR expressed as a percentage of the total heart weight after in vitro ischaemia-reperfusion in the rat. Data was analysed using a one-way ANOVA; data shown is mean ± SEM; n= 5-7.

Figure 4.5 shows that treatment with $17\beta E_2$ tended to reduce the necrotic zone in comparison to placebo after *in vitro* ischaemia-reperfusion, although this failed to reach significance (P=0.057). Treatment with MPA alone did not significantly alter the necrotic zone relative to placebo. There was however, a significant ($P<0.05$) increase in the necrotic area within the AAR in the group treated with $17\beta E_2$ and MPA in combination, compared to treatment with $17\beta E_2$ alone (Figure 4.5).

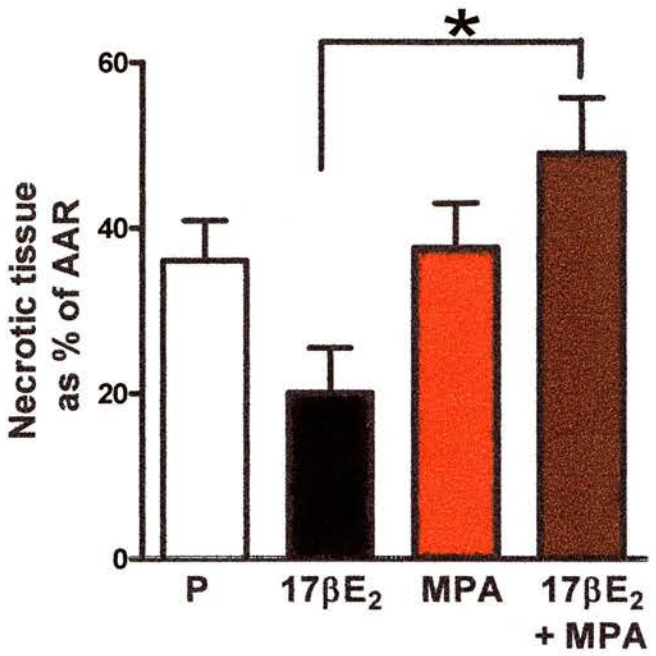


Figure 4.5. **The necrotic myocardium after *in vitro* ischaemia-reperfusion.**
*The necrotic area of the myocardium, expressed as a percentage of the AAR, of the rat heart after ischaemia-reperfusion in vitro. Data was analysed using a one-way ANOVA and a Bonferroni post-hoc test; data shown is mean ± SEM; *P<0.05; n=5-7.*

There was no significant difference between treatment groups in the coronary perfusion pressure before the onset of ischaemia (Table 4.4). There was a drop in perfusion pressure at the onset of ischaemia, this was only significant in the placebo treated group ($P<0.05$; Table 4.4). The perfusion pressure then recovered during ischaemia to pressures similar to those before ischaemia. The pressure then remained relatively constant throughout reperfusion. None of the treatment groups altered these characteristic changes in perfusion pressure throughout ischaemia and reperfusion (Table 4.4).

Treatment	Before ischaemia	Ischaemia onset	Reperfusion	2hrs Reperfusion
Placebo	64.1 ± 4.4	38.9 ± 2.6#	47.6 ± 4.6	46.5 ± 4.4
17βE$_2$	64.1 ± 9.4	44.8 ± 7.7	51.1 ± 6.8	45.4 ± 8.5
MPA	76.0 ± 8.1	47.8 ± 3.4	63.5 ± 6.6	63.4 ± 8.6
17βE$_2$ + MPA	92.0 ± 12.4	62.7 ± 8.7	72.4 ± 12.3	78.8 ± 20.5*

Table 4.4. **Mean perfusion pressure of the rat myocardium through out *in vitro* ischaemia reperfusion.**

*Mean perfusion pressure in mmHg of the rat myocardium through out the period of in vitro ischaemia-reperfusion on the Langendorff. The data was analysed using a repeated measures ANOVA within groups and a one-way ANOVA between groups at each time point. The ANOVA was followed by a Bonferroni post-hoc test. Data shown is mean ± SEM; *P<0.05 compared to placebo at the same time; #P<0.05 compared to before ischaemia; n= 5-7.*

4.5. Discussion

The main aim of this chapter was to investigate the effect of MPA on the cardioprotective effects of $17\beta E_2$ in a model of ischaemia-reperfusion. The results of this chapter demonstrate that the ability of chronically administrated $17\beta E_2$ to protect rat hearts from ischaemia-reperfusion injury *in vivo* and *in vitro* is inhibited by the co-administration of MPA. The $17\beta E_2$ mediated decrease in necrotic area *in vivo* and *in vitro* as well as the attenuated neutrophil infiltration are antagonised by the co-administration of MPA.

MPA is a synthetic progesterone. MPA is used in HRT because it has increased bioavailability and is metabolised slower than progesterone (Mathrubutham *et al.* 1981), thus lower doses are required. The slower metabolism is due to an additional methyl and acetate group, which means that MPA has to be hydrolysed prior to metabolism and that it is a poorer substrate for 5-alpha reductase. Progesterone is also a precursor to other steroids, the synthetic analogue does not act as a precursor. Therefore when MPA is administrated hormones downstream are not unintentionally increased (Ottoson *et al.* 1984).

Large scale prospective double-blinded studies, such as the WHI (The Women's Health Initiative Study Group 1998), HERS (Hulley *et al.* 1998; discussed in Section 1.9) and 'Estrogen Replacement and Atherosclerosis' (ERA) trial (Herrington *et al.* 2000) have failed to demonstrate cardiovascular benefits of HRT regimes in post-menopausal women (Langer 2002). Such findings were not expected and contradicted those obtained from observational studies of ERT and experimental results of $17\beta E_2$ in the cardiovascular system (Bush *et al.* 1987; Falkeborn *et al.* 1992; Henderson *et al.* 1991). These discrepancies lead to a hypothesis that progestogens may inhibit the beneficial properties of E. Such a hypothesis has lead to a number of studies that have investigated the potential interactions between E and progestogens (Adams *et al.* 1997; Gorodeski *et*

al. 1998; Koh *et al.* 2004; McNeill *et al.* 2002; Minshall *et al.* 1998; Oishi *et al.* 2004; Simoncini *et al.* 2004).

In the current study myocardial injury following ischaemia and reperfusion was reduced in ovx rats that were treated with $17\beta E_2$ compared to those receiving placebo. Previous studies have demonstrated the protective effects of $17\beta E_2$ in experimental models of myocardial ischaemia-reperfusion (Kolodgie *et al.* 1997; Squadrito *et al.* 1997; Zhai *et al.* 2000). The proposed mechanisms for $17\beta E_2$ mediated protection in this model are discussed previously in Section 3.5. However, when co-administered with MPA, the $17\beta E_2$ induced cardioprotective properties were clearly inhibited. The amount of necrotic tissue after ischaemia-reperfusion in MPA and $17\beta E_2$ treated rats was comparable to the placebo treated rats.

The reduced levels of MPO activity within the myocardial tissue after treatment with $17\beta E_2$ indicates that there was an attenuated inflammatory response and neutrophil infiltration. This finding is in agreement with previous observations that $17\beta E_2$ attenuates neutrophil infiltration (Squadrito *et al.* 1997). The $17\beta E_2$ mediated decrease in neutrophil infiltration was negated when MPA and $17\beta E_2$ were co-administered. The increase in MPO activity, almost reached placebo levels, thus suggesting the inhibition of neutrophil infiltration into the AAR by $17\beta E_2$ was impaired by MPA. However, in this model attenuated neutrophil infiltration is clearly not the only mechanism sufficient to explain the protective effects of $17\beta E_2$.

In isolated buffer perfused hearts from rats receiving $17\beta E_2$ there was a tendency for myocardial injury to be reduced compared to rats receiving placebo. The protective properties displayed by $17\beta E_2$ *in vitro* were not as marked as *in vivo*. The advantage of using an *in vitro* ischaemia-reperfusion set up is discussed in Section 2.2.2.2. The anti-oxidant and vasodilator properties of E may have an important influence here (Babiker *et al.* 2002). The tendency for $17\beta E_2$ to decrease the *in vitro* myocardial damage in this

study, suggests that *in vivo* mechanisms such as neutrophil infiltration, adhesion and activation play a significant role in the protection displayed by E but that these work in synergy with *in vitro* mechanisms. The co-administration of $17\beta E_2$ and MPA *in vitro* led to a significantly greater necrotic area than that in rats treated with $17\beta E_2$ alone. This result demonstrates that MPA must also be having an effect on the other protective mechanisms of E that we see *in vitro*. These results *in vitro* depend upon the hormone treatment having an effect upon the tissue during the period prior to perfusion as the perfusate did not contain any hormones.

Interestingly MPA did not prevent all systemic effects of E. While body weight was characteristically reduced in E treated rats (Beckett *et al.* 2002), MPA had no influence on body weight either alone or in combination with E. Similarly, MPA had no influence on the uterine weight. E stimulated the proliferation of uterine epithelial cells (Bigsby *et al.* 2004), both in the absence and presence of MPA. MPA has previously been reported to act systemically and to prevent these effects of E (Di Carlo *et al.* 1984; McNeill *et al.* 2002). However, the present findings suggest that the level of MPA administered in this study was only sufficient to inhibit E in the heart, suggesting that a more sensitive mechanism is involved. In this study a control group of rats were treated with MPA alone, this treatment did not have a significant effect on any of the parameters measured in this study.

Several mechanisms have been proposed to account for the interactions between progestogens and other steroid hormones. The androgenic progestogen, MPA, was recently shown to impair E receptor signalling in endothelial cells (Simoncini *et al.* 2004), leading to a reduction in its ability to increase NO bioavailability (Oishi *et al.* 2004). MPA also has antagonistic properties at glucocorticoid receptors, leading to an increased ability of human leukocytes to express the adhesion molecule ICAM-1 (Simoncini *et al.* 2004). The binding of E to ERs is also reported to be inhibited by MPA (Di Carlo *et al.* 1983) and progestogens like MPA are reported to down regulate ER in target tissues (Chwalisz *et al.* 1998; Di Carlo *et al.* 1984). Complete inhibition of E

binding to its' receptors seems an unlikely explanation for the observations in the present study as this would also have limited the effects of $17\beta E_2$ on the body weight and uterine proliferation, unless ERs in cardiovascular target tissue are down regulated at a lower concentration of MPA than that required in other target tissues. Impaired receptor signalling and interaction with other steroid receptors that may co-operate with E are attractive alternative hypotheses. A reduction by MPA on the ability of E to increase NO bioavailability, would lead to impairment of the vasodilatory and anti-inflammatory effects of E in this ischaemia-reperfusion injury model. Indeed, progestogens have previously been shown to interfere with E induced enhancement of coronary flow (Gorodeski *et al.* 1998) and endothelium-dependant dilation (Chataigneau *et al.* 2004; Wakatsuki *et al.* 2001). Inhibition of neutrophil infiltration would also be enhanced by MPA via reduced NO availability and also by increased expression of adhesion molecules like ICAM-1. More recently MPA has been shown to act through the progesterone receptor within the CVS to inhibit the beneficial properties of E (Iruela-Arispe *et al.* 1996; Karas *et al.* 2001; Mercuro *et al.* 1999; The writing group for the PEPI trial 1995). Such binding within this study would account for the attenuated cardiovascular effects but the unabated systemic effects on body and uterine weight.

Previous studies of this sort have used either more (Levine *et al.* 1996; Oishi *et al.* 2004) or less (Mensah-Brown *et al.* 2004) MPA compared to E. For this initial study we decided to use equal amounts to investigate the impact of MPA on E's protection of the myocardium. In the WHI 4x more MPA than E was administered. However, the results in this chapter have demonstrated that at these concentrations the effects of E on the myocardium are more sensitive to MPA than its effects on body weight and uterine weight. The dose of MPA administered is clearly influential, Hanke et al demonstrated that the ability of $17\beta E_2$ to attenuate atherosclerotic plaque development in rabbits was inhibited by high doses of a progesterone but not at lower doses (Hanke *et al.* 1996). There is also evidence that lower doses of both E and progestogens are more beneficial and eliminate some of the detrimental effects, particularly increased thrombogenesis (Stevenson 2000). New clinical trials such as the 'Kronos Early Estrogen Protection

Study' (KEEPS) that has commenced recently is investigating the use of lower hormone doses the dosing regime of this study is 0.459mg.day^{-1} of E and 200mg of a progesterone for only 12 days of the month (Harman *et al.* 2005).

It is clear from this study that MPA, when co-administered *in vivo* with E has the ability to negate the protective effects of E in the myocardium. These data further suggest that interactions between E and progestogens, like MPA, can lead to inhibition of Es' anti-inflammatory effects and enhanced inflammatory cell accumulation, as well as enhancement of other synergistic mechanisms that protect the tissue more directly from ischaemic injury.

CHAPTER 5

THE ROLE OF OESTROGEN DURING REMODELLING POST-MYOCARDIAL INFARCTION IN FEMALE MICE.

5.1. Introduction

It is surprising that pre-menopausal women are reported to have higher acute mortality following MI compared to age-matched men, this is discussed in Section 1.8 (Vaccarino *et al.* 1999). Also mentioned in Section 1.8, ovx female mice supplemented with $17\beta E_2$ have increased mortality in the acute period following MI (Sharif 2002; van Eickels *et al.* 2003) and impaired remodelling (Smith *et al.* 2000) compared to those supplemented with placebo.

In the mouse model of MI, the major cause of acute sudden death is rupture of the LV free wall at the infarct border (Cavasin *et al.* 2003). The incidence of this cardiac rupture is increased by $17\beta E_2$ in ovx female mice (Sharif 2002; van Eickels *et al.* 2003). This observation suggests that $17\beta E_2$ might interfere with the process of infarct healing that eventually results in formation of a collagen rich scar and stabilisation of the infarct site. The process of infarct healing involves inflammatory cell infiltration, ECM degradation by MMPs, angiogenesis, myofibroblast proliferation and collagen synthesis as described in chapter 1 (Blankesteijn *et al.* 2001; Lindsey *et al.* 2002). Cardiac rupture begins at 3 days after MI (Figure 5.1). Therefore the disrupted remodelling is likely to primarily involve the earliest stages of remodelling prior to myofibroblast proliferation around four days post-MI, collagen synthesis and angiogenesis around seven days after MI.

Several studies have linked MMP activity to the incidence of rupture, and in particular activity of MMP-2 and MMP-9 (Ducharme *et al.* 2000; Hayashidani *et al.* 2003). At the time of rupture it is plausible that the MMPs are at there most active, playing a major role in the breakdown of the ECM and consequently fatal rupture. E is known to regulate MMP activity in the reproductive tract (Marbaix *et al.* 1992) and non-reproductive tissues, such as vascular smooth muscle and tumour cells (Potier *et al.* 2001; Wingrove *et al.* 1998). However, little is known about the influence of E on MMPs or the principal regulators of their activity, the TIMPs (Spinale 2002) in the heart. The extent of the initial inflammatory response has also been suggested as a determinant of the incidence of cardiac rupture post-MI (Cavasin *et al.* 2003).

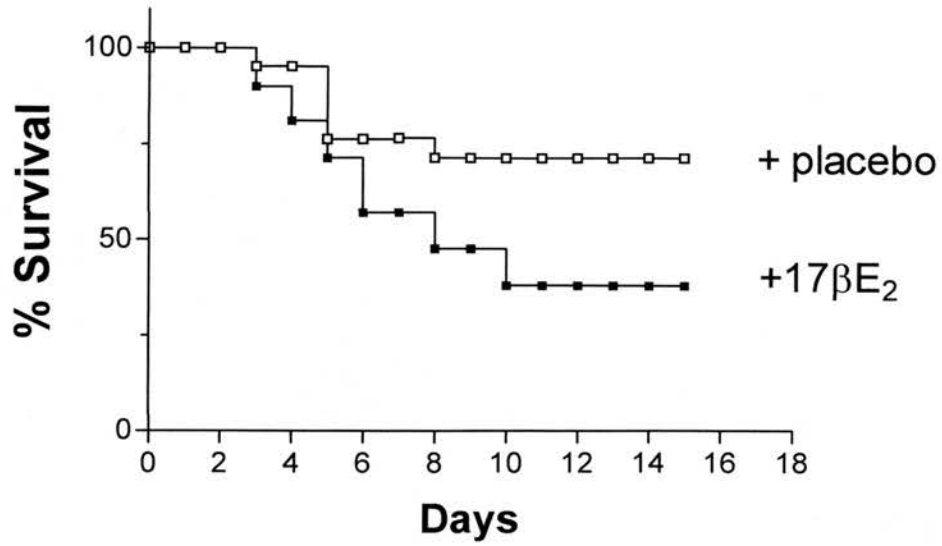


Figure 5.1. **Kaplan-Meier survival plot.**
The mortality rate in ovx mice treated with 17βE_2 after MI is significantly greater than in mice receiving placebo after ovx. The significant increase in mortality is observed from day 3-8 and is due to cardiac rupture. Mice surviving up to day 10 post-MI have a much greater chance of survival and are unlikely to suffer fatal cardiac rupture. Logrank test, P=0.04, n=21.(Previous studies in our lab; Sharif 2002)

5.2. Aims

The aim of the present study was to investigate if 17βE_2 modifies the early infarct healing and remodelling post-MI and if this leads to an increase in the incidence of cardiac rupture.

To investigate the effect of 17βE_2 on the remodelling process neutrophil infiltration and myocardial necrosis were measured at 2 days after MI, this is when neutrophil infiltration is at a maximum and therefore most likely to be of importance within the remodelling. The expression and activity of MMPs and TIMPS were measured at 4 days after MI. We hypothesised that due to a higher incidence of cardiac rupture in mice around this time point that the MMP activity would be at a maximum then and that this level would be most relevant.

5.3. Methods

5.3.1. Hormone Administration

Female mice (n=59) aged 13 weeks, were prepared for surgery as described in Section 2.2. The mice then underwent ovx as described in Section 2.2.1.1 and were randomly assigned to receive placebo or 17βE_2 pellets subcutaneously at the nape of the neck. The mice were allowed to recover as detailed in Section 2.2.1.1.

5.3.2. Myocardial Infarction

One week after ovx, MI was induced in the 14 week old female mice. The MI procedure is outlined in detail in Section 2.2.1.2.

5.3.3. Determination of infarct size and necrotic cell death

Two days after MI the mice (n=12) were anaesthetised as detailed in Section 2.2.1.2. The heart was removed from the chest and processed using TTC analysis as described in Section 2.3.1 without Evans blue dye.

5.3.4. Determination of apoptotic cell death and neutrophil infiltration

The apoptotic cell death and myocardial neutrophil infiltration were also recorded at two days post-MI. At the time of harvesting the tissue, the mice (n=15) were anaesthetised as described in Section 2.2.1.2. The hearts were removed from the chest and rinsed in water before being weighed and cut in half transversely from apex to base. The hearts were then processed for immunohistochemistry analysis as described in Section 2.4.2.1.

Apoptotic cell death was analysed using a TUNEL detection kit (Promego, UK), as described in Section 2.3.2. Neutrophil infiltration was quantified by immunohistochemistry. Haematoxylin and eosin staining was used initially to identify the cells present in the infarct, this protocol is explained in Section 2.4.2.2. To further analyse and quantify the neutrophil infiltration into the ischaemic myocardium a specific

neutrophil marker, GR-1 antibody was used in immunohistochemistry. This protocol is detailed in Section 2.4.2.3.

5.3.5. MMP and TIMP analysis

Four days after MI the mice (n=32) were sacrificed as described in Section 2.2.1.2. The myocardium was sectioned and cultured for 24 hours in culture medium, this protocol is detailed in Section 2.2.1.2. After 24 hours the culture medium was freeze-dried and stored, as described in detail in Section 2.2.1.2. MMP activity was analysed using gelatin zymography, detailed in Section 2.5.5. The activity of TIMPs was quantified using reverse gelatin zymography, this protocol is detailed in section 2.5.4. The expression of MMP-13 and TIMP-2 was analysed and quantified using western blotting, detailed in Section 2.5.3, with specific antibodies (Appendix 2).

5.4. Results

5.4.1. Mortality rate post myocardial infarction

In the initial study with an end point of 2 days post-MI, for the investigation of neutrophil infiltration and apoptotic cell death there were no premature deaths. Throughout the 4 day time course of the second study experimental MI caused premature death in 22% of the placebo group and 38% of the $17\beta E_2$ group (P=NS).

5.4.2. Necrotic and apoptotic tissue post myocardial infarction

Histological analysis of mouse hearts taken 2 days post-MI showed a significant increase in infarct size compared to control operated mice. The infarct size was determined by the weight of necrotic tissue, this significant increase was significantly reduced with administration of $17\beta E_2$, compared to placebo treated mice ($P<0.001$; Figure 5.2a). $17\beta E_2$ treatment also decreased the number of TUNEL positive cells in the infarct compared to placebo treated mice, suggesting suppression of apoptosis ($P<0.001$; Figure 5.2b).

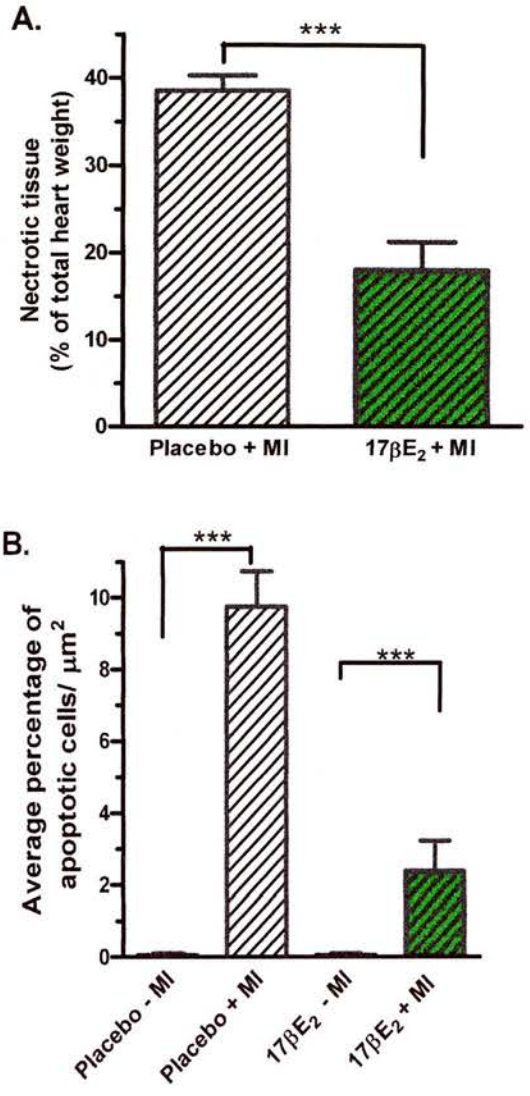


Figure 5.2. **Necrotic tissue and apoptotic cells 2 days post myocardial infarction.** ***(A.)*** *The necrotic area of the mouse myocardium 2 days post-MI or control surgery. The necrotic area was assessed using tri-phenyltetrazolium chloride. The data shown is expressed as a percentage of the total heart weight.* ***(B.)*** *Histogram of TUNEL staining, showing the percentage of apoptotic cells within the infarcted myocardium. Ten areas within the infarct were assigned at random and the number of TUNEL positive cells averaged for each heart The data shown is mean ± SEM and was analysed using an* ***(A.)*** *unpaired two tailed t-test and* ***(B.)*** *two-way ANOVA with a Bonferroni post hoc, **P<0.001, n=4-5 per group.*

5.4.3. Neutrophil infiltration post myocardial infarction

Two days after MI neutrophils were detected in the infarcted LV through haematoxylin and eosin staining. Figure 5.3 highlights the presence of neutrophils in the infarcted myocardium compared to the non-infarcted RV. For quantifying the neutrophils within the infarcted LV a specific immunohistochemistry approach was taken using a GR-1 antibody.

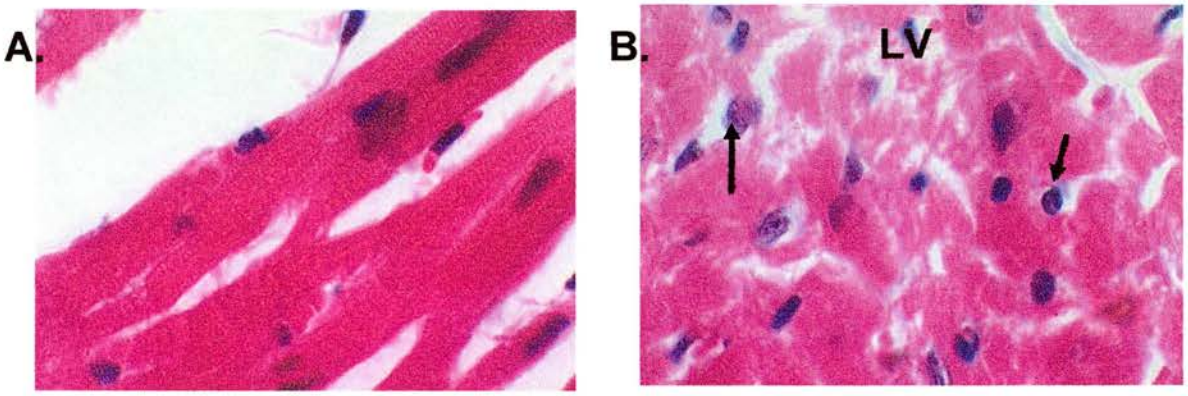


Figure 5.3. **Haematoxylin and eosin stain of infarcted myocardium.**
Haematoxylin and eosin stain of ***(A.)*** *the RV and* ***(B.)*** *the infarcted LV 2 days after myocardial infarction. The heart is from a mouse that had previously had an ovx and implantation of placebo pellet. There are no neutrophils visible in the RV, in the LV the arrows highlight some of the neutrophils present.*

Immunohistochemistry using a specific GR-1 antibody clearly illustrated the presence of neutrophils within the infarcted LV (Figure 5.4). Very few GR-1 positive cells were visible in control-operated hearts or in the RV of infarcted hearts. Treatment with $17\beta E_2$ significantly ($P<0.05$) reduced the number of neutrophils infiltrating into the infarcted region of the myocardium compared to the placebo treated mice (Figure 5.4).

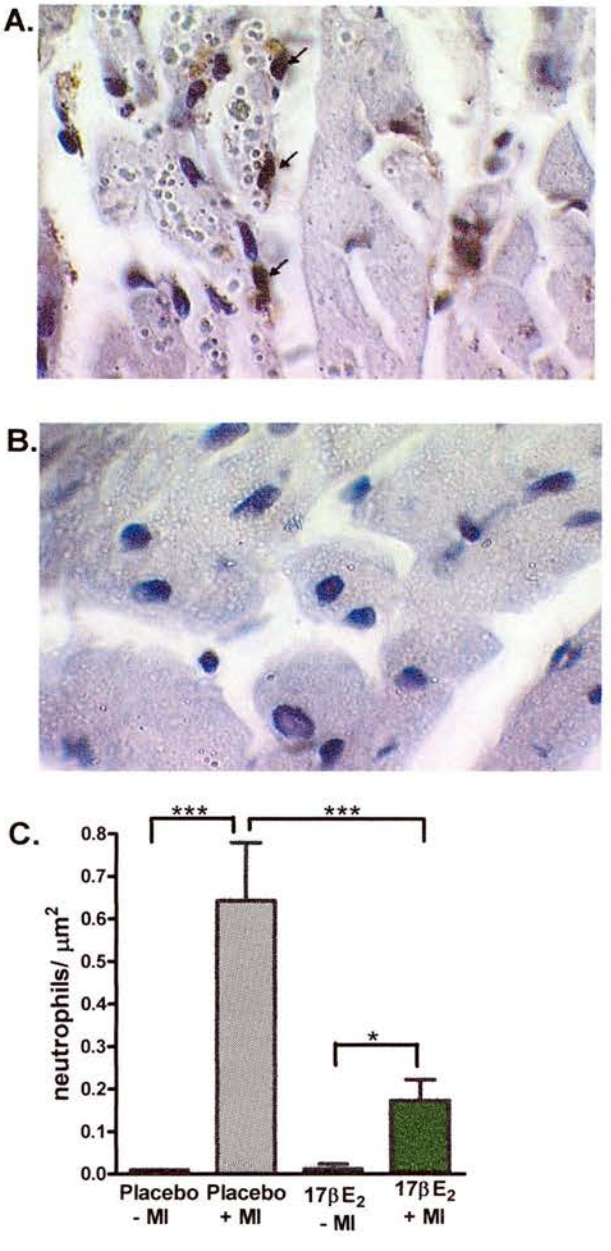


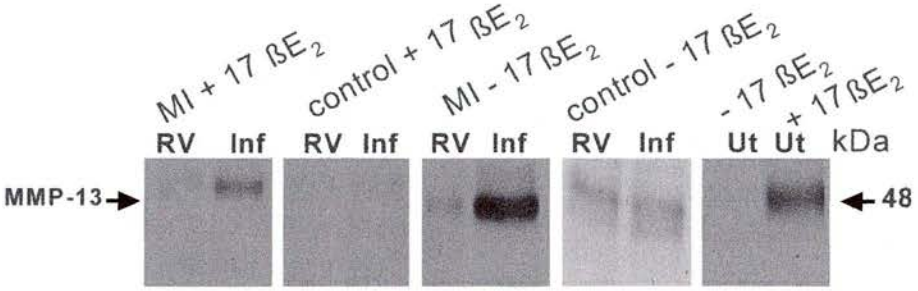
Figure 5.4. **Neutrophil infiltration post myocardial infarction**
Neutrophil infiltration in the infarcted LV 2 days post-MI. GR-1 immunohistochemistry staining of the infarcted region of ***(A)*** *placebo treated mice and* ***(B)*** *17βE$_2$ treated mice, with arrows denoting GR-1 positive cells.* ***(C)*** *Neutrophil infiltration into the left ventricle (LV) of infarcted mice ± 17βE$_2$ treatment. Data shown is mean ± SEM, expressed per µm^2 as viewed at x1000 magnification. The data was analysed using a two-way ANOVA with a Bonferroni post hoc, ***P<0.0001; n=4 per group.*

5.4.4. Detection of MMP-13 expression

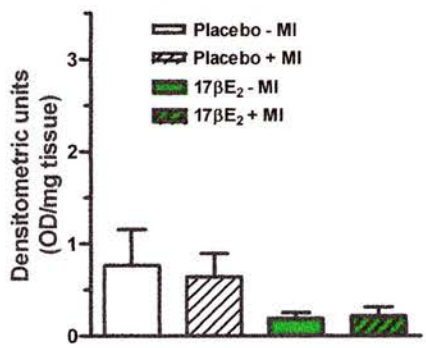
Western blot analysis (Figure 5.5a) demonstrated a low basal expression of MMP-13 protein in control hearts and in the non-infarcted RV four days post-MI (Figure 5.5b). Expression of MMP-13 in the infarct zone was significantly increased four days following MI surgery (Figure 5.5c; $P<0.05$). This increase in MMP-13 expression following MI was significantly reduced in mice receiving $17\beta E_2$, such that levels were comparable to those in placebo treated non-infarcted hearts (Figure 5.5c). Interestingly, treatment with $17\beta E_2$ also reduced MMP-13 expression in control hearts. In contrast to the heart, expression of MMP-13 in the uterus was significantly increased by treatment with $17\beta E_2$ relative to placebo ($P<0.01$; Figure 5.5d).

Figure 5.5. **The influence of MI and $17\beta E_2$ on secreted MMP-13 from myocardial tissue and uterus.**
(A.) *A typical Western blot showing MMP-13 expression, in reducing conditions in the RV or the region corresponding to the infarcted LV (Inf) of ovx MI and control operated mice receiving either placebo or $17\beta E_2$. The expression of MMP-13 in the uterus of mice receiving placebo or $17\beta E_2$ was used as in internal control. MMP-13 corresponds to a protein band ~48 kDa. Histograms of the relative densitometric values for MMP-13 expression in the* ***(B.)*** *right ventricle,* ***(C.)*** *infarct and* ***(D.)*** *uterus. Data shown is the mean $\pm$ SEM; it was analysed using a two-way ANOVA followed by a Bonferroni post-hoc for* ***B*** *and* ***C****; unpaired two-tailed t-test for* ***D****; *P<0.05 **P<0.01 (n=5).*

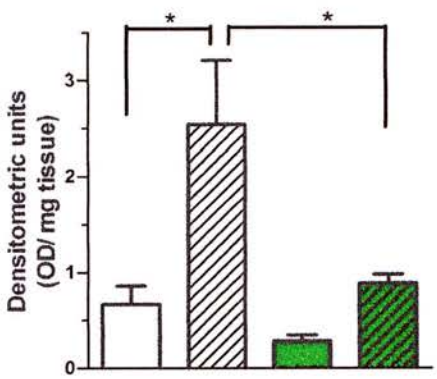
A.


MI + 17 ßE2
control + 17 ßE2
MI - 17 ßE2
control - 17 ßE2
- 17 ßE2
+ 17 ßE2
RV Inf RV Inf RV Inf RV Inf Ut Ut kDa
MMP-13
48

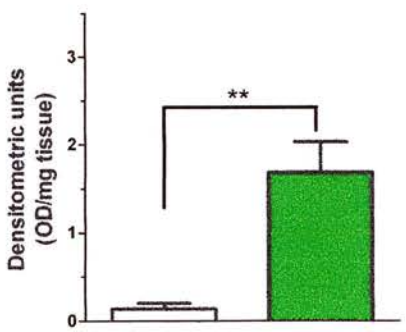
B.


Placebo - MI
Placebo + MI
17βE2 - MI
17βE2 + MI
Densitometric units (OD/mg tissue)
0
1
2
3

C.


*
*
Densitometric units (OD/ mg tissue)
0
1
2
3

D.

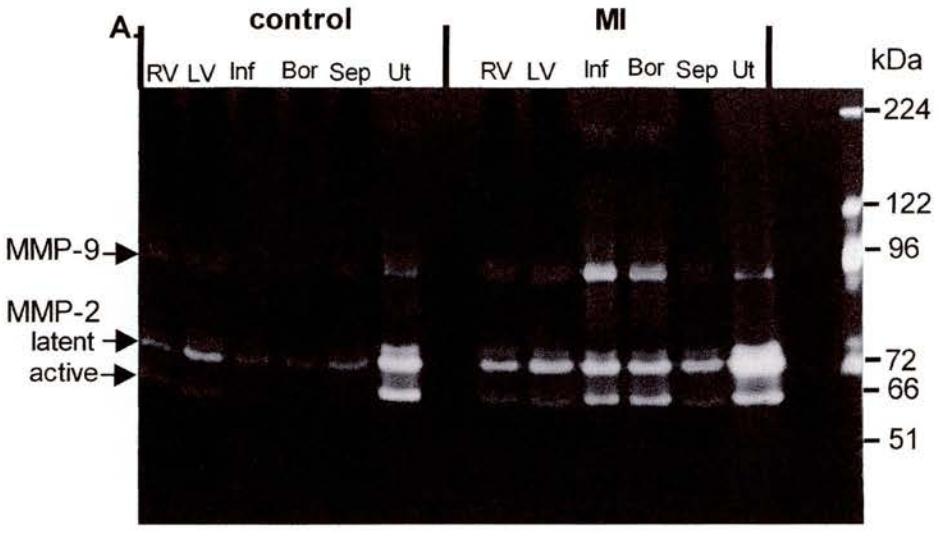

**
Densitometric units (OD/mg tissue)
0
1
2
3

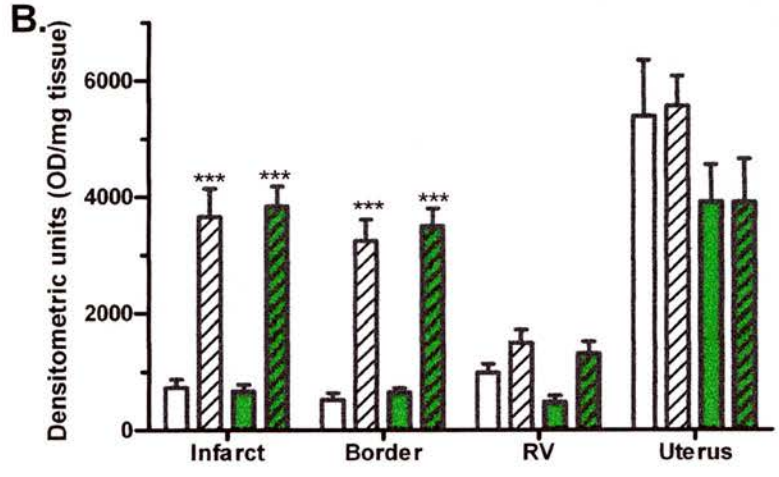
5.4.5. Detection of MMP-2 and MMP-9 activity

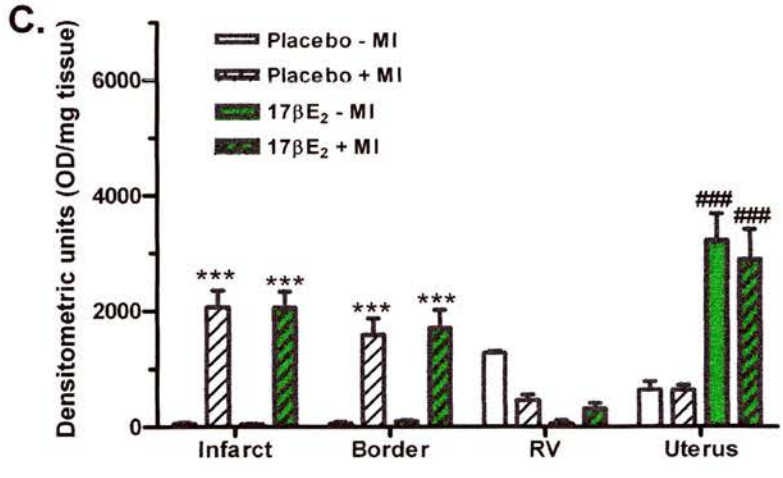
Gelatin zymography of culture medium conditioned by regions from all hearts showed the presence of bands of gelatinase activity at 92 kDa, 72 kDa and 66 kDa, corresponding to latent MMP-9, latent and active MMP-2, respectively (Figure 5.6a). Following MI, the secreted enzymatic activity of MMP-2 significantly ($P<0.001$) increased 3-4 fold in the I and B zones of the myocardium relative to comparable areas in control hearts and to the non-infarcted RV of the same heart (Figure 5.6b). There was a more marked, 10-20 fold increase in secreted activity of MMP-9 in the I and B zones compared to control hearts and to the RV of the same heart ($P<0.001$; Figure 5.6c).

Treatment of mice with $17\beta E_2$ had no significant effect on the secreted activity of MMP-2 or MMP-9 in any area of control hearts and did not alter the activity in MI hearts (Figure 5.6b and c). In the uterine tissue from the same animals, $17\beta E_2$ had no influence on secreted MMP-2 activity (Figure 5.6b) but consistently increased secreted MMP-9 activity ($P<0.001$; Figure 5.6c).

Figure 5.6. **The influence of MI and $17\beta E_2$ on secreted MMP-2 and MMP-9 activity.**
(A) *A typical gelatin zymogram showing secreted MMP-2 and MMP-9 activity from the right ventricle (RV), non-infarcted left ventricle (LV), infarcted LV (Inf), septum (Sep) and uterus (Ut) from mice 4 days post-MI or equivalent regions from control mice. Gelatinase activity at 92 kDa, 72 kDa and 66 kDa corresponds to MMP-9 and to latent and active forms of MMP-2 respectively. The influence of $17\beta E_2$ on the activities of* ***(B.)*** *MMP-2 and* ***(C.)*** *MMP-9 secreted from the infarct, infarct border, RV or uterus of C57B6J/129sv at 4 days post MI or in corresponding regions from control hearts and uterus. Data is corrected for tissue weight and is shown as mean $\pm$ SEM. The data was analysed using a two-way ANOVA followed by a Bonferroni post-hoc test ***$P<0.001$, compared to the control group; n=6-8 per group.*


A.
control
MI
RV LV Inf Bor Sep Ut
RV LV Inf Bor Sep Ut
kDa
224
122
96
72
66
51
MMP-9
MMP-2
latent
active


B.
Densitometric units (OD/mg tissue)
6000
4000
2000
0
***
Infarct
Border
RV
Uterus


C.
Placebo - MI
Placebo + MI
17βE2 - MI
17βE2 + MI
Densitometric units (OD/mg tissue)
6000
4000
2000
0
***
###
Infarct
Border
RV
Uterus

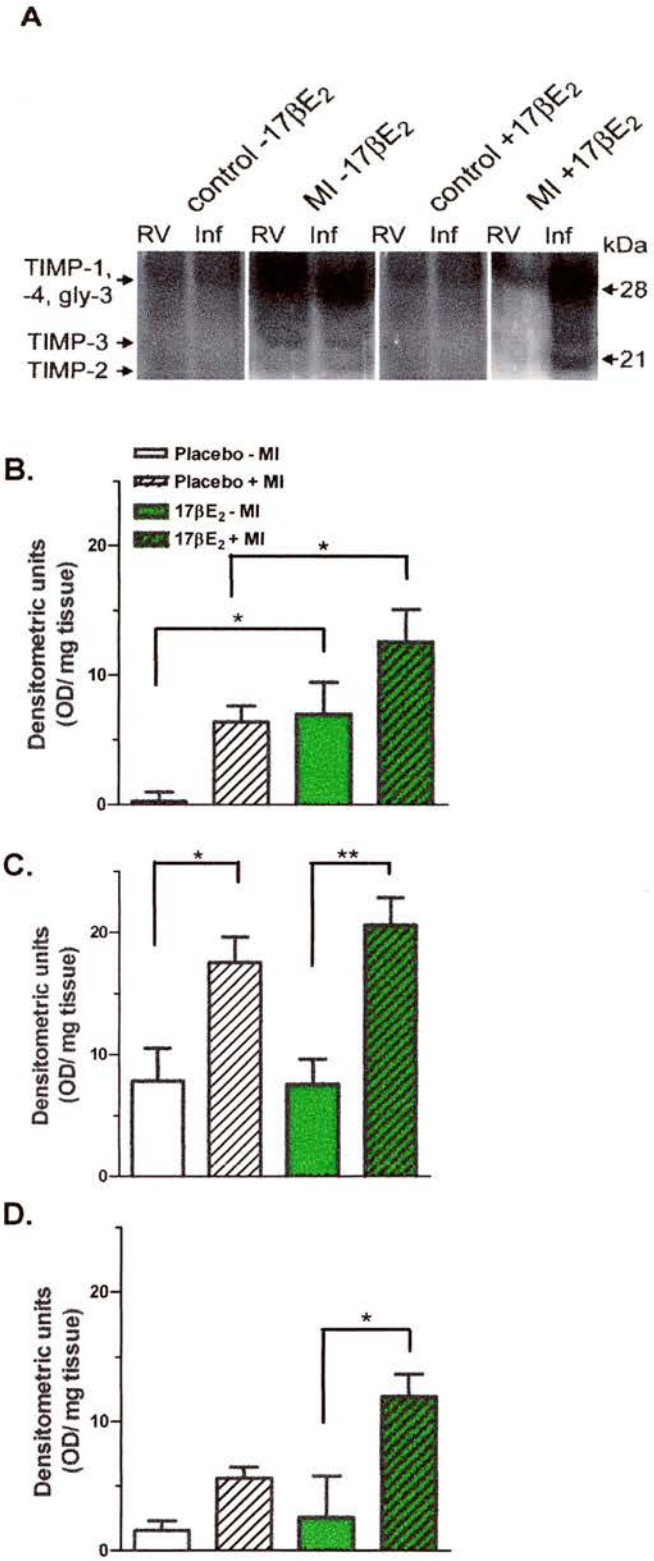
5.4.6. Detection of TIMP activity and expression

Reverse gelatin zymography demonstrated secreted activity of TIMP-1, glycosylated TIMP-3 and TIMP-4 at ~28 kDa, TIMP-2 activity at ~21 kDa and unglycosylated TIMP-3 activity at ~24 kDa (Figure 5.7a). The secreted activity of TIMP-1, glycosylated TIMP-3 and TIMP-4 could not be individually resolved, due to their close molecular weights (28, 27 and 30 kDa respectively). Following MI there was a significant ($P<0.05$) increase in the activity of all TIMPs secreted from the infarct zone compared to control operated mice (Figure 5.7), this increase in activity was particularly marked for TIMP-2 (Figure 5.7b). This was confirmed by Western blots and a trend for increased TIMP-2 protein expression after MI compared to control operated mice (Figure 5.9).

Treatment with $17\beta E_2$ significantly ($P<0.05$) increased secreted TIMP-2 activity in the infarct zone of MI mice and the corresponding area of control mice compared to placebo treated mice (Figure 5.7b). However, $17\beta E_2$ did not alter the expression of TIMP-2 protein within the infarct zone of MI or the corresponding area of control mice (Figure 5.9). The increased unglycosylated TIMP-3 activity post-MI was enhanced in the presence of $17\beta E_2$ (Figure 5.7d), while $17\beta E_2$ had no significant effect on the summated activity of TIMP-1, glycosylated TIMP-3 and TIMP-4 (Figure 5.7c).

In the uterus all four TIMPs showed a trend towards increased secreted activity when treated with $17\beta E_2$ compared to placebo treated animals, although only TIMP-2 activity was significantly enhanced ($P<0.05$; Figure 5.8).

Figure 5.7. **TIMP activity after myocardial infarction.**
(a.) *A typical reverse zymmogram of TIMP –1, glycosylated -3 and –4 activity at ~28 kDa, unglycosylated TIMP -3 at ~26 kDa and TIMP-2 at ~21 kDa, in the right ventricle (RV), infarct (Inf), border (Bor) and uterus (Ut) after myocardial infarction. The mice had previously undergone ovx ± 17βE$_2$ supplementation. Histogram of the relative activity of* ***(b.)*** *TIMP-2,* ***(c.)*** *TIMP-1, glycosylated TIMP-3 and TIMP-4 and (d.) unglycosylated TIMP-3. Data shown is mean ± SEM, Two-way ANOVA followed by Bonferroni post-hoc *P<0.05, **P<0.01 (n=6-8).*


A
control -17βE2
MI -17βE2
control +17βE2
MI +17βE2
RV Inf RV Inf RV Inf RV Inf
kDa
TIMP-1, -4, gly-3
TIMP-3
TIMP-2
28
21
B.
Placebo - MI
Placebo + MI
17βE2 - MI
17βE2 + MI
Densitometric units (OD/ mg tissue)
C.
D.

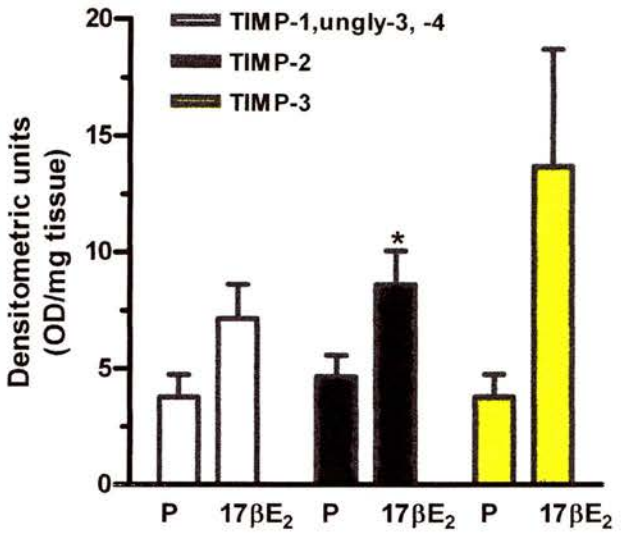


Figure 5.8. **The influence of $17\beta E_2$ on secreted TIMP activity from the uterus**
*The secreted activity of TIMP-1, unglycosylated TIMP-3 and TIMP-4, TIMP-2 and TIMP-3 from the uterus after ovx and placebo or $17\beta E_2$ treatment. The data is corrected for tissue weight and is shown as relative densitometric units; mean ± SEM and was analysed using a paired two-tailed t-test and a Bonferroni post hoc test for each TIMP; *P<0.05 compared to placebo; n=4-8.*

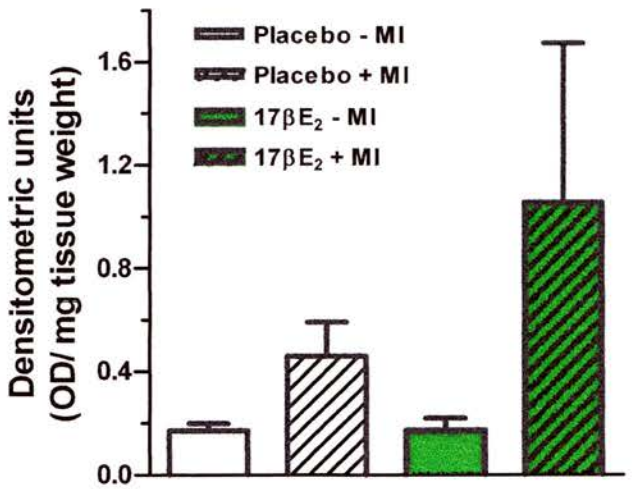


Figure 5.9. **TIMP-2 expression after myocardial infarction**
The influence of MI and $17\beta E_2$ on TIMP-2 expression in the left ventricle measured by Western blot. Data is corrected for tissue weight and is shown as relative densitometric units, mean ± SEM. The data was analysed using an unpaired two-way ANOVA; n=3.

5.5. Discussion

In this chapter we aimed to investigate the influence of $17\beta E_2$ on myocardial remodelling post-MI. The results from this chapter show that $17\beta E_2$ suppresses cell necrosis, apoptosis, inflammation and MMP-13 activity in the infarcted region of the heart post-MI. The activity of MMP-2 and MMP-9 post-MI is not however reduced in parallel with suppression of cell death and inflammation in $17\beta E_2$-treated mice. The activity of all the TIMPs is increased post-MI. TIMP-2 activity is increased after $17\beta E_2$ treatment in animals having undergone MI or not. An exaggerated stimulus for matrix degradation and disrupted cardiac remodeling may lead to destabilization of the forming scar and result in death due to rupture.

The murine model of MI is characterised by the occurrence of sudden death due to cardiac rupture, occurring between 3-8 days after MI (Gao *et al.* 2005; Tao *et al.* 2004). To date studies of rupture have linked its incidence to larger infarcts (Gao *et al.* 2005), an enhanced inflammatory response and subsequent MMP activation post-MI (Cavasin *et al.* 2004; Sun *et al.* 2004). Male gender is also associated with an increased likelihood of rupture (Gao *et al.* 2005) and this has been attributed to the presence of testosterone (Cavasin *et al.* 2003; Cavasin *et al.* 2004; Cavasin *et al.* 2006). Recently, it has been shown that chronic administration of $17\beta E_2$ significantly enhances the risk of rupture following MI in female mice (Sharif 2002; Smith *et al.* 2000; van Eickels *et al.* 2003), although the reasons for this are unclear. The aim of the present study was to determine if $17\beta E_2$ regulates the ECM degradation during the acute remodelling post-MI.

The initial identification of neutrophils in the infarcted myocardium with haematoxylin and eosin staining enabled a good indication of the cells present at two days post-MI. The staining with haematoxylin and eosin was not sufficiently clear for the quantification of neutrophil infiltration into the infarct. The subsequent use of a neutrophil specific, GR-1 antibody, detailed in Section 2.4.2.3 with haematoxylin stain

provided clearer identification of neutrophils within the infarct. The clearer identification of neutrophils enabled their infiltration to be quantified.

Infarct size is reduced in mice treated with $17\beta E_2$ relative to placebo treated controls. We have shown that following MI $17\beta E_2$ reduced the necrotic area by on average 53 percent. This is in agreement with previous reports (van Eickels *et al.* 2003), this is at least partly due to a reduction in apoptotic cardiomyocyte death, an effect of $17\beta E_2$ that has recently been attributed to activation of phospo-ser^{423}-Akt and ERK 1/2 (Patten *et al.* 2006; Vilatoba *et al.* 2005), this is likely to be through a non-genomic pathway. The inflammatory response, indicated by the presence of GR-1 positive cells, was also suppressed in the infarct of mice treated with $17\beta E_2$ compared to placebo. This correlates with effects of $17\beta E_2$ in previous studies of both acute ischaemia-reperfusion (in the preceding chapters) and chronic MI (Cavasin *et al.* 2004). Inhibition of NFκB transcription (Evans *et al.* 2001) and enhancement of endothelial cell expression of Fas ligand are thought to contribute to reduction of neutrophil infiltration by $17\beta E_2$ (Amant *et al.* 2001). Previous studies in both animals and humans show that topical application of E results in accelerated wound healing (Ashcroft *et al.* 1999), with characteristics similar to those described during the early infarct healing phase. In the myocardium, wound healing is reported to be more efficient in normally cycling females following MI than in males (Cavasin *et al.* 2004). It may be that chronic elevation of E in the present study results in formation of a scar that is unable to adapt to the constant pressure changes, resulting in rupture. Assessing the collagen and more specifically the collagen cross-linking within the infarct area would provide an indication to the stability of the infarct. In this model of MI collagen is typically observed from day 7 onwards (Small *et al.* 2005). Therefore, the samples in this study that were collected at 2 days post MI did not show any sign collagen when stained with van Geison (data not shown). This would be a good study to carry out for a better understanding of the scar formed, in terms of the tensile strength, rigidity and possibly how an increased collagen turnover alters these parameters, post-MI.

E can influence the activity of matrix degrading enzymes in non-cardiac tissues (Helvering *et al.* 2005; Potier *et al.* 2001). Given the clear evidence that MMP activity is a determinant of the extent of rupture post-MI in the mouse myocardium (Ducharme *et al.* 2000; Hayashidani *et al.* 2003; Heymans *et al.* 1999; Tao *et al.* 2004), it seemed probable that changes in matrix degrading activity by $17\beta E_2$ may also influence scar stability in the heart.

Expression of MMP-13 is constitutively low in the myocardium, but is increased by four days after MI, when it is likely to be secreted by proliferating fibroblasts (Xiaoquang Liu 1997). Treatment of mice with $17\beta E_2$ decreases the MI associated increase in MMP-13, and this may suggest suppression of fibroblast proliferation by $17\beta E_2$. However, the decrease in MMP-13 expression was also observed in control mice, demonstrating that $17\beta E_2$ has additional direct effects on MMP-13 expression, that are independent of MI (Lu *et al.* 2006).

In the present study, the secreted activities of MMP-2 and MMP-9 were significantly increased after MI in the infarct and infarct border, in agreement with earlier studies (Heymans *et al.* 1999; Tao *et al.* 2004). Increased MMP-9 activity has been shown to coincide temporally with neutrophil infiltration and it is expressed in neutrophils and macrophages found in the infarct region at four days post-MI (Heymans *et al.* 1999; Lindsey *et al.* 2001). MMP-2 is constitutively expressed within the heart and has a role in ECM turnover. Infiltrating inflammatory cells also express MMP-2 and are a likely source of the increased activity post-MI in the mouse myocardium (Hayashidani *et al.* 2003; Tao *et al.* 2004). Hearts from animals treated with $17\beta E_2$ had a significant reduction in neutrophil infiltration following MI. Given the apparent importance of these cells as a source of gelatinase activity, it was surprising that there was no consequent reduction in MMP-2 or MMP-9 activity in these hearts. Myocytes can also express gelatinases in the heart post-MI and these are likely to be an alternative source of MMP-2 and MMP-9 activity in the present study (Romanic *et al.* 2001). The evidence linking gelatinase activity to the incidence of cardiac rupture is very strong, based on studies

using inhibitors and KO mouse models (Ducharme *et al.* 2000; Hayashidani *et al.* 2003; Heymans *et al.* 1999). However, in the present study, MMP-2 and MMP-9 activity was not modified by $17\beta E_2$, suggesting that this is not the pathway primarily responsible for its enhancement of cardiac rupture. Recently, Nahrendorf *et al* have also observed a decreased inflammatory response with a concomitant increase in MMP-9 protein in the myocardium post-MI, and this was also associated with an increase in the incidence of rupture (Nahrendorf *et al.* 2006). The inflammatory response is likely to be important during the initial remodelling period for the removal of necrotic tissue.

TIMPs exert largely un-discriminated inhibition on MMP activity, preventing complete degradation of the ECM, therefore the activity of both TIMPs and MMPs is vital for ECM maintenance. MI resulted in a significant increase in the activity of all four known TIMPs in the infarct region (Cleutjens *et al.* 2002; Peterson *et al.* 2000). Treatment with $17\beta E_2$ had no influence on expression of TIMP-2, as shown by Western blotting, but stimulated a further increase in its activity within the infarct zone. Activity of TIMP-3 was also enhanced by E. Previous studies have reported that TIMP-2 activity is enhanced via stimulation of ERK 1/2 pathway (Munshi *et al.* 2004). Interestingly, E is known to increase the expression of ERK 1/2 in mice (Vilatoba *et al.* 2005), it is likely this could be a mechanism of regulating TIMP-2 activity in the myocardium. The impact of increased TIMP-2 and -3 activity after $17\beta E_2$ treatment on scar stability is unclear and likely to depend on the complexes formed with MMPs. While TIMPs are inhibitors of MMP activity, recent evidence has emerged suggesting that TIMP-2 can also activate pro-MMP-2 into the active enzyme (Baker *et al.* 2002; Rapti *et al.* 2006). TIMPs also have functions in cells that are independent of their action upon MMPs (Lovelock *et al.* 2005). TIMP-2 and –3 might also influence wound healing through their ability to directly enhance fibroblast proliferation and differentiation; there is an increase in fibroblasts at the infarct border post-MI (Lovelock *et al.* 2005).

The results of this study confirm that $17\beta E_2$ has cardioprotective properties related to reduction of cell apoptosis and inflammation in the acute phase following MI. The mechanisms for this protection are discussed previously in Section 3.4. However, despite this, administration of $17\beta E_2$ can result in destabilisation of the ischaemic zone, rendering it prone to rupture. The data demonstrate that enhancement of matrix degradation by MMPs cannot account for this effect of $17\beta E_2$. In fact the weight of evidence supports suppression of overall MMP activity by $17\beta E_2$. Thus the mechanism of rupture is distinct from that previously reported in males (Cavasin *et al.* 2003; Cavasin *et al.* 2004; Cavasin *et al.* 2006). Previous experimental studies of MI in mice have reported a higher systolic BP in males who also have a higher rate of LV rupture compared to normal cycling females (Cavasin *et al.* 2004). In a previous study we have shown that the current dose of $17\beta E_2$ administered to ovx mice increases the BP compared to ovx females receiving placebo, therefore it is possible that the haemodynamics post-MI have a significant effect on the rate of rupture (Ness 2005).

It is clear from clinical and experimental studies that E can have long-term benefit in slowing progression to heart failure (Beer *et al.* 2006; Smith *et al.* 2000). The reduction in infarct size and inflammatory response reported here will contribute to this protection in animals that do not succumb to rupture.

CHAPTER 6

GENERAL DISCUSSION

6.1. General Discussion

Due to the observed lower incidence of CVD in pre-menopausal women compared to their age-matched men and post-menopausal women E was proposed to protect against CVD. These suggestions were supported by the first observational clinical trials (Stampfer *et al.* 1985), but yet results from randomised controlled clinical trials suggest that HRT treatment is not beneficial within the cardiovascular system and even increases the risk of a cardiovascular event in the first year (Hulley *et al.* 1998; Rossouw *et al.* 2002). Data recorded on in-hospital mortality also suggests that pre-menopausal women are more likely to succumb to detrimental remodelling in the acute period after MI (Vaccarino *et al.* 1999). Numerous animal models have reported the cardioprotective properties of E within the vasculature and heart (Smith *et al.* 2000; Squadrito *et al.* 1997; Williams *et al.* 1992).

At the time of starting this research, shortly after the WHI clinical trial results were published, limited research had been carried out on the effect of progesterone analogues on the cardiovascular properties of E (Hanke *et al.* 1996; Wakatsuki *et al.* 2001). During the course of my research extensive analysis has been carried out on the data obtained from the WHI trial (Grimes *et al.* 2002; Herrington *et al.* 2003). The WHI and similar clinical studies are now widely accepted but the experimental design continues to be scrutinised and the results raising specific questions that may help direct existing hormone treatment in the most appropriate way to women who will benefit most. Chief amongst these is the age of recipients, the delay in commencing HRT and the cardiovascular health at the time of commencing treatment. Sub-group analysis has made it apparent that younger females benefit from HRT whilst older women with more advanced CHD have no benefit or fare worse (Grodstein *et al.* 2006). The average age of the study population in the WHI and HERS was 60-65 years and all had been post-menopausal for at least 6 months. Re-analysis of the 'nurses health study' has provided evidence that most benefit from HRT is seen in younger women (Manson *et al.* 2006) and sub-group analysis of the WHI ERT arm shows a strong trend in cardioprotection in the 50-59 years age group (Manson *et al.* 2003; Salpeter *et al.* 2004). Young women

with POF have endothelial dysfunction that is normalized with hormone replacement treatment (Kalantaridou *et al.* 2006). Age and CHD are associated with endothelial dysfunction (Seals *et al.* 2006), experimental studies have shown reduced ER expression in such arteries (Losordo *et al.* 1994; Post *et al.* 2003). E is also known to be detrimental in the latter stages of atherosclerotic plaque development (ESHRE Capri workshop group 2006). Experimental studies utilising animals of different ages at the time of ovx and commencing E replacement at various time points after ovx would help to associate these differences with the results seen in clinical trials. The studies carried out in this thesis only looked at animals at one age and all of these received the replacement hormones at the time of ovx. In our investigation using mature animals, we demonstrated that MPA reversed the $17\beta E_2$ mediated reduction in necrotic tissue and neutrophil infiltration. These experimental results demonstrate possible mechanisms for the failure of clinical HRT studies. A recent clinical trial, The 'Early Versus Late Intervention Trial with Estradiol' (ELITE) is designed to specifically investigate the differences between peri- and post-menopausal women, it will run for 5 years, reporting in 2010 (ELITE 2005).

The dose and route of E administration are also increasingly recognised as being of importance in determining the response to hormone replacement. There is now some evidence that a lower dose of E than that used in some of the earlier trials may reduce a number of the detrimental effects seen in previous trials, particularly increased thrombogenesis (Stevenson 2004). The WHI and HERS trials both used oral administration of their hormone regimes. However, transdermal administration may be more physiological and preferable. Transdermal application avoids the first pass effects in the liver, providing more constant blood levels and importantly a more physiological, pre-menopausal estradiol:estrone ratio (Sturdee 1999). Administration by the transdermal route has been shown to avoid the increased risk of thromboembolism (Post *et al.* 2003) and pro-inflammatory state that have been associated with oral administration (Lakoski *et al.* 2005; Manson *et al.* 2006), whilst apparently retaining the beneficial effects of E on endothelial function (Blumel *et al.* 2003). The experimental

studies carried out in this thesis all used subcutaneous hormone replacement, this is the most common method of hormone replacement after experimental ovx and could be a reason for the discrepancies observed between experimental and clinical trials. The controlled double-blind study KEEPS, is designed to test the importance of the route of administration. The study is designed to run for five years, until 2010 and include women between the age of 40 and 55 years old (Kronos 2006).

The cellular mechanisms of E within the CVS have also received enhanced interest since the clinical trial results (Dubey *et al.* 2004; Manson *et al.* 2003). Improved knowledge of which ER E acts through to mediate it's cardioprotective effects may help us target specific receptors to attain the desired benefit from pharmacological methods. Experimental studies in genetically-modified mice have provided some confusing information, this could be due to a large degree of variability between species and disease models (Mendelsohn 2002). For example, E is known to be cardioprotective following injury and this has been variously attributed to both ERα (Wang *et al.* 2006) and ERβ (Gabel *et al.* 2005; Korte *et al.* 2005). The genetic difference between mouse species is not only evident in experimental studies of $17\beta E_2$ but also studies of myocardial remodelling and MMP expression and activity (Gao *et al.* 2005). For example, MMP-2 mRNA is decreased in C57/Bl6/J aortic VSMC after treatment with E, yet is increased in C3F1/HeJ VSMC. The effect on MMP-9 mRNA expression was also different in the two cell lines; E decreased MMP-9 in C3F1/HeJ VSMC but had no effect in C57/Bl6/J VSMC MMP-9 activity (Shi *et al.* 2003). The genetic variability limits the relevance of studies in mice and the ability to correlate them to humans.

At the time of commencing this thesis the ER responsible for E mediated protection and the attenuated inflammatory response had not been identified. Due to the limited availability of selective ER agonists the majority of research into the individual ERs had been carried out in KO mice (Gabel *et al.* 2005; Karas *et al.* 1999; Korte *et al.* 2005; Wang *et al.* 2006). Increased availability of selective ER agonists and the development of novel ER antagonists has enabled increased research into the role of individual ERs

including their function in other experimental animals, such as rats and rabbits (Booth *et al.* 2005; Pelzer *et al.* 2005). This is the first time that a novel ER antagonist has been used to investigate the role of individual ERs within the myocardium. In the initial investigation into the role of individual ERs after ischaemia-reperfusion we found that ERα mediates the cardioprotective properties of $17\beta E_2$. We were able to show this through the use of a novel ERβ antagonist Org44488, administered to rats treated with $17\beta E_2$. We then confirmed these results with the use of a novel ERα agonist Org37445. The protection obtained with $17\beta E_2$ was similar to that obtained in previous studies using the same model (Squadrito *et al.* 1997). Acute administration of an ERα agonist was recently shown to mediate reduced cell death during ischaemia-reperfusion in rabbits (Booth *et al.* 2005). Our study demonstrated the chronic effects of ERα stimulation *in vivo* and *in vitro,* which enabled us to investigate the combined genomic and non-genomic protection displayed by $17\beta E_2$ in the presence and absence of neutrophils. In the current studies we were unable to identify which pathways were responsible for the displayed protection.

Understanding the role of individual ERs within the CVS is unlikely to result in ER specific compounds being used in humans because the side effects would be too large. The ability to activate ERs in selective tissues is now possible through the selective stimulation of co-activators with 'selective oestrogen receptor modulators' (SERMs). SERMs exhibit some tissue specificity in their effects. For example, raloxifene has similar properties to E in the CVS such as lowering of LDL and improvement of endothelial function, while being antagonistic to Es proliferative effects in the uterus and breast. Clinical trials to date using SERMs have resulted in conclusions of a null effect on CVD, yet again the age of administration could be the key (Barrett-Connor *et al.* 2006; Blumenthal *et al.* 2004; Mosca *et al.* 2001; Somunkiran *et al.* 2006).

In contrast to the protective properties of $17\beta E_2$ during acute ischaemia-reperfusion, it is associated with increased detrimental events after chronic MI. Prior to commencing this

thesis we (Sharif 2002) and others (van Eickels *et al.* 2003) had reported that experimentally mice receiving $17\beta E_2$ compared to placebo prior to MI have a higher mortality rate due to cardiac rupture. MMP-2 and MMP-9 activity had previously been linked to increased cardiac rupture within the LV (Ducharme *et al.* 2000; Hayashidani *et al.* 2003). The relevance of $17\beta E_2$ during cardiac rupture and the remodelling period preceding it had not been studied previously. Since starting this thesis the activity and expression of MMPs and TIMPs during this intense remodelling period have been investigated (Tao *et al.* 2004). The conclusions drawn from our studies are that $17\beta E_2$ disrupts myocardial remodelling through decreased neutrophil infiltration and an attenuated initial inflammatory response. Treatment with $17\beta E_2$ also resulted in a decreased necrotic area and MMP-13 expression but did not have any effect on MMP-2 and MMP-9 activity. Despite these apparent protective mechanisms the detrimental inflammatory response may be more dominant in the remodelling period and lead to the observed significant elevated levels of cardiac rupture. The MMP activity is clearly not the only factor in cardiac rupture. Controversially, experiments that have compared the mortality between normal cycling female and male mice have reported an increased rupture rate in males and a detrimental effect of testosterone compared to E (Cavasin *et al.* 2004). In the study by van Eickels, which also showed $17\beta E_2$ to increase cardiac rupture the ovx mice receiving $17\beta E_2$ had plasma E_2 levels of 81 ± 13 $pg.ml^{-1}$; like our study, this is at the high end of the physiological range. The discrepancy in these results may therefore be due to the continuous high levels of $17\beta E_2$ compared to the non-ovx cyclic females with lower plasma E_2 levels. E_2 may become detrimental at continuous high plasma levels. This discrepancy is consistent with a speculation of the failure of the clinical trails of HRT, which provided continuous levels of hormones. Prior to the menopause the plasma level of E in females is cyclical. Like HRT treatment, experimental models using ovx animals and subcutaneous $17\beta E_2$ pellets maintain a continuous plasma E level. Mice supplemented with $17\beta E_2$ used in this study and others may be limited in their physiological relevance due to the continuous high level of circulating E. The plasma E level obtained in the current study using mice was in the

physiological range, although it was at the high end of pro-oestrus levels. For mice to have such high levels of E continuously is not physiological. The plasma E levels obtained in the rats during this thesis were closer to mid-cycle physiological levels. In our experimental study the rats received $17\beta E_2$ replacement immediately after ovx and natural E deprivation. The $17\beta E_2$ administrated at a constant level in our studies was cardioprotective. When MPA was co-administered the protective properties of $17\beta E_2$ were lost, studies that use various doses of $17\beta E_2$ and MPA would determine if the dose of hormone has a significant effect on myocardial protection, as it does in atherosclarotic plaque development (Hanke *et al.* 1996).

Gender differences in neutrophils could also account for some of the discrepancies, there are significantly fewer neutrophils detected in female mice compared to male mice (Tao *et al.* 2004). This finding is in agreement with the studies within this thesis. Increased macrophage expression in the mouse model of MI is typically observed from around day four onwards when it temporally co-localised with MMP-2 (Tao *et al.* 2004); interestingly there are fewer macrophages detected at this time point in female mice compared to males (Cavasin *et al.* 2004). We did not study the infiltration of macrophages in the infarct region because we were interested in the neutrophil infiltration which occurs earlier and therefore did not leave the mice four days post-MI when immunohistochemistry analysis was carried out. It would be interesting to see if the same pattern of infiltration is true in the ovx mice and whether this is a key difference in the remodelling process.

The finding that the AAR was consistent between all the treatment groups in the different studies, suggests that the experimental procedure was reliable and reproducible, and cannot therefore be accountable for the observed differences in the necrotic tissue between treatment groups. The lack of significant differences between the haemodynamics of the treatment groups means that these can also be eliminated for causing any significant effect on the observed myocardial differences and outcomes of ischaemia-reperfusion.

Limitations of the studies performed in this thesis include the low blood blood pressure and heart rate in chapter 4. The low haemodynamics are likely to be due to over anaesthetising the rats, a reason for his could be that the Sagatal was going off. When Sagatal goes off the animals take longer to go under and therefore anaesthetic top-ups are more likely to be administered. A further limitation in this study involves the administration of the ERα agonist in the comparison to the $17\beta E_2$ treatment. Treatment with $17\beta E_2$ is administered continuously after ovx and elimination of endogenous E, in comparison ERα is administered after a period of E deprivation and placebo treatment. A direct comparison group for the ERα group would involve implanting the $17\beta E_2$ pellet after a period of total E deprivation. The wide range of effects $17\beta E_2$ has within the CVS (Mendelsohn *et al.* 1999) makes the interpretation of results difficult to interpret and exact mechanisms to be pin-pointed. The use of KO models has limited physiological relevance due to wide spread compensatory mechanisms; for this reason traditional pharmacological inhibitors are able to give a better understanding.

Further work developing the findings from this thesis would provide interesting results and continue to enlighten us into the cellular mechanisms of selective ERs. Exploiting the use of selective agonists, it is necessary to administer a chronic ERβ agonist to rats and analyse the effect this has on the outcome of ischaemia-reperfusion. This experiment would complete work within this thesis to distinguish between the roles of individual receptors within the myocardium during injury. In light of experiments such as Jakowski and others that demonstrate E alters ER expression I think it would be interesting to examine the expression of both ERα and ERβ before and after ischaemia-reperfusion in the rat myocardium.

In the current thesis we only investigated the effect of $17\beta E_2$ on the myocardial remodelling post MI. Utilising the current model to asses the properties of collagen (type of collagen and extent of cross-linking) and the fibroblasts in the infarct and at the border around 4-7 days after MI would also help us understand how $17\beta E_2$ is altering the

wound healing and scar formation. It would be interesting to utilise the selective ER agonists and antagonist in this model and find out which ER the effects of E are being mediated through in this model. Recently ERα was reported to suppress MMP-13 promoter activity in a rabbit cell line (Lu *et al.* 2006), it'll be interesting to see if this is the case in the myocardium. The use of MMP inhibitors in conjunction with E in the murine model of MI will also provide some more indication into the role of E in their regulation and the importance of this in light of the present findings.

The overall aim of this thesis was to gain better understanding into the mechanisms and interaction of E in the heart. In summary, our findings are that ERα mediates the *in vivo* and *in vitro* $17\beta E_2$ mediated decrease in neutrophil infiltration, oxidative stress and cell death in the rat model of ischaemia-reperfusion. We also show that the $17\beta E_2$ mediated attenuation of cell death and neutrophil infiltration after ischaemia-reperfusion is attenuated by co-administration of MPA. Finally we demonstrate that $17\beta E_2$ disrupts the acute remodelling process post-MI. The disruption results in detrimental remodelling and an increased incidence of cardiac rupture. These results may be attributed to the anti-inflammatory properties of $17\beta E_2$ that beneficially protect against ischaemia-reperfusion. Thus, the same mechanism that protects the myocardium from ischaemia-reperfusion maybe attributable for the enhanced cardiac rupture observed in mice acutely after MI.

APPENDIX

Appendix 1

1.1 Krebs Henseleit buffer

Component	Conentration
NaCl	118.0 mM
KCl	4.7 mM
$NaHCO_3$	25.0 mM
KH_2PO_4	1.17 mM
$MgSO_4 \cdot 7H_sO$	1.2 mM
$CaCl_2$	3.0 mM
EDTA	0.5 mM
D-glucose	11 mM

pH 7.4; filtered through waterman paper.

1.2 Solutions for measurement of myocardial infarct size

i. Evan's blue dye

Component	Concentration
Evan's blue dye	1% w/v (in 0.9% saline)

ii. 2,3,5-triphenyltetrazolium

Component	Concentration
2,3,5-triphenyltetrazolium (TTC)	1% w/v (in 0.9 % saline)

iii. Formalin (10%)

Component	Concentration
40 % formalin	10 ml
NaCl	9 % w/v

Made up in 90 ml dH_2O.

1.3 Buffers and solutions for extraction of MPO from rat heart tissue and neutrophils

i. Homogenisation Buffer I (0.02M Phosphate buffer)

Component	Concentration
NaCl	0.1 M
NaH_2PO_4	0.02 M
NaEDTA	0.05 M
DH_2O	200 ml

Adjust to pH 7.4 with NaOH

ii. Homogenisation Buffer II (0.05M Phosphate buffer)

Component	Concentration
Na_2HPO_4	0.05 M
NaH_2PO_4	0.05 M
DH_2O	200 ml

Make up Na_2HPO_4 in 4ml and add to 196 ml NaH_2PO_4. Adjust to pH 5.4 with NaOH

iii. Citrate Phosphate buffer (0.05 M)

Component	Concentration
Citric acid	0.05 M 7 ml
Na_2HPO_4	0.05 M 13ml

Adjust to pH 5.0

iv. Reaction buffer

Component	Concentration
o-dianisidine dihydrochloride	0.05 M
H_2O_2	0.015 % v/v
0.05 M citrate phosphate buffer	20 ml

1.4 Immuno staining solutions

i. Acid Alcohol

Component	Volume
Conc HCl	1ml
Absolute alcohol	350ml
DH_2O	149ml

ii. Scotts tap water

Component	Concentration
K_2CO_3	0.01 M
$MgSO_4$	0.17 M

Electrophoresis buffers

1.5 Gelatin Zymography/ Reverse Zymography

i. Sample Application Buffer

Component	Concentration
Glycerol	20 % w/v
SDS	2.0 % w/v
Bromophenol blue	0.04 % w/v

ii. Running tank buffer (x10)

Component	Concentration
Tris	0.25 M
Glycine	1.9 M
SDS	1.0 %

iii. Gelatin Zymography Wash buffer TBS (x10)

Component	Concentration
Tris	0.5 M
NaCl	1.5 M

Adjust to pH 8.0 with 5N HCl

iv. Reverse Zymography Wash buffer

Component	Concentration
Tris	50 mM
$CaCl_2$	5 mM
Triton-X-100	2.5 %

Adjust to pH 7.5 with 5N HCl

v. Gelatin Zymography Triton X-100 wash

2.5 % Triton X-100 in TBS 1:10

vi. Gelatin Zymography Digestion buffer

Component	Concentration
Tris	50 mM
NaCl	0.2 mM
$CaCl_2$	5.0 mM
$ZnCl_2$	1.0 μM
Brij-35	0.02 %

Adjust to pH 7.6 with 5N HCl

vii. Reverse Zymography Digestion Buffer

Component	Concentration
Tris	50 mM
$CaCl_2$	5 mM

Adjust to pH 7.5 with 5N HCl

viii. Destaining solution

Component	Concentration
Methanol	30 %
Glacial acetic acid	10 %
Water	60 %

ix. Staining solution

0.5 % w/v Coomassie Brilliant Blue R250 in destaining solution

1.6 Western Blot

i. Sec-butanol

Equal volumes of sec-butanol and distilled water (50 ml) were shaken for 3 minutes to mix them. The solution was then allowed to settle (approximately 3hrs) prior to use. The top layer is used to layer on the top of gels whilst they polymerise, forming a straight top. This prevents a wavy line (if water alone is used) or the butanol drawing the water from the gel (if butanol is used alone).

ii. Sample application Buffer

Component	Concentration
Tris	1.5 % w/v
SDS	4.0 % w/v
Dithiothretiol (DTT)	2.0 % w/v
Bromophenol blue	0.05 % w/v

Adjust to pH 6.75 with 5N HCl, stored at -20°C

iii. Running tank buffer (x10)

Component	Concentration
Tris	0.25 M
Glycine	1.9 M
SDS	1.0 %

iv. Transfer buffer

Component	Concentration
Tris	25 mM
Glycine	192 mM
Methanol	20 % v/v

pH 8.0-8.4

v. Stock Wash Buffer TBS (x10)

Component	Concentration
Tris	0.5 M
NaCl	1.5 M

Adjust to pH 7.4 with 5N HCl

vi. TTBS Wash buffer (as used)

Tween 20 0.05 % v/v min TBS x1

vii. Solution for diluting primary antibody

Component	Volume
TTBS 5% BSA	9.85ml
NaN_3 (2.5%)	100µl
Antibody	50µl

viii. Stripping solution

Component	Concentration
Glycine	0.2 M
SDS	1 %

1.7 Gel Composition

Zymography

i. Resolving gel 7.5 % (for 2 gels 0.75 mm thick)

Component	Volume
ddH_2O	3.85 ml
Tris 1.5 M pH 8.8	2.5 ml
Gelatin 10 mg/ml	1.0 ml
10 % SDS	100 µl
10 % ammonium persulphate	50 µl
Acrylamide/Bis	2.5 ml
TEMED	5 µl

N.B Gelatin was dissolved in the microwave prior to addition.

ii. Stacking gel 4% (for 2 gels 0.75 mm thick)

Component	Volume
ddH_2O	3.05 ml
Tris 0.5 M pH 6.8	1.25 ml
10 % SDS	50 µl
10 % ammonium persulphate	50 µl
Acrylamide/Bis	0.65 ml
TEMED	5 µl

Reverse Zymography

iii. Resolving gel 12 % (for 2 gels 0.75 mm thick)

Component	Volume
ddH_2O	1.69 ml
Tris 1.5 M pH 8.8	2.5 ml
Gelatin 10 mg/ml	1.0 ml
10 % SDS	100µl
10 % ammonium persulphate	50 µl
Acrylamide/Bis	4.0 ml
Solution A	0.66 ml
TEMED	5 µl

iv. Stacking gel 5% (for 2 gels 0.75 mm thick)

Component	Volume
ddH_2O	2.89 ml
Tris 0.5 M pH 6.8	1.25 ml
10 % SDS	50 µl
10 % ammonium persulfate	50 µl
Acrylamide/Bis	0.812 ml
TEMED	5 µl

Western blot

v. Resolving gel

Component	Volume (7.5%)	Volume (12%)
ddH_2O	9.7 ml	6.7 ml
Tris 1.5 M pH 8.8	5.0 ml	5.0 ml
10 % SDS	200 µl	200 µl
10 % Ammonium persulphate	100 µl	100 µl
Acrylamide/Bis	5.0 ml	8.0 ml
TEMED	10 µl	10 µl

vi. Stacking gel 4% (for 2 gels 1.5mm thick)

Component	Volume
ddH_2O	6.1 ml
Tris 0.5 M pH 6.8	2.5 ml
10 % SDS	100 µl
10 % Ammonium persulphate	100 µl
Acrylamide/Bis	1.3 ml
TEMED	10 µl

1.8 Aminopropylethoxysaline (TESPA) slide coating

Bathe slides for 10 seconds each in the following solutions:

1. 10% HCl in 70% ethanol
2. DEP H_2O
3. 100% acetone

Air dry the slides, then take through the following, 10 seconds each:

4. 2% TESPA in acetone
5. 100% acetone
6. 100% acetone

Slides are then air dried again and can be stored in an air tight container for up to one month.

LIPPINCOTT
WILLIAMS & WILKINS
A Wolters Kluwer Company

September 27, 2006

Helen Jeanes
35/2 Cockburn Street
Edinburgh
Mid Lothian
EH1 1BP
UK

VIA EMAIL TO: h.l.jeanes@sms.ed.ac.uk September 27, 2006

FEE: NONE

RE: Jeanes, H.L. Wanikiat, P. Sharif, I. Gray, G.A.
Menopause 2006;13(1):80-86

USE: Thesis

CONDITION OF AGREEMENT

Permission is granted upon the return of this signed agreement to Lippincott Williams & Wilkins (LWW). Please sign and date this form and return to:

Lippincott Williams & Wilkins
David O'Brien, Worldwide Copyright Management
351 W Camden Street, 4 North
Baltimore, MD 21201
USA

Permission is granted and is subject to the following conditions:

1) A credit line will be prominently placed and include the journal article author and article title, journal title, volume number, issue number, and the inclusive pages.
2) The requestor warrants that the material shall not be used in any manner, which may be derogatory to the title, content, or authors of the material or to LWW.
3) Permission is granted for use only as specified in your correspondence. Rights herein do not apply to future reproductions, editions, revisions, or other derivative works.
4) Permission granted is non-exclusive, and is valid throughout the world in the English language.
5) LWW cannot supply the requestor with the original artwork or a "clean copy".
6) Permission is valid if the borrowed material is original to a LWW imprint (Lippincott-Raven Publishers, Williams & Wilkins, Lea & Febiger, Harwal, Igaku-Shoin, Rapid Science, Little Brown & Company, Harper & Row Medical, American Journal of Nursing Co., and Urban & Schwarzenberg-English language.)

Requestor accepts: _____ Date: _____

Menopause: The Journal of The North American Menopause Society
Vol. 13, No. 1, pp. 80-86
DOI: 10.1097/01.gme.0000196593.44335.eb

Medroxyprogesterone acetate inhibits the cardioprotective effect of estrogen in experimental ischemia-reperfusion injury

Helen L. Jeanes, BSc,[1*] Payong Wanikiat, PhD,[1,2*] Isam Sharif, PhD,[1] and Gillian A. Gray, PhD[1]

Abstract

Objective: Results from recent clinical trials of estrogen and progestogen therapy (EPT) suggest that some progestogens may interfere with the cardiovascular benefits of estrogen (E). The aim of this study was to investigate whether medroxyprogesterone acetate (MPA) modifies the protective effect of E in experimental ischemia-reperfusion (IR) injury in vivo and in vitro in the rat.

Design: Ovariectomized female Wistar rats (250-280 g, n = 61) received E, MPA, E and MPA, or placebo subcutaneously. Fourteen days later, hearts were isolated and perfused with Krebs Henseleit for in vitro experiments or left in situ for in vivo experiments. In both cases, the left coronary artery was occluded for 45 minutes, followed by 2 hours of reperfusion.

Results: In vivo E significantly reduced the necrotic zone of reperfused hearts (21.8% ± 1.7% of area at risk) compared with placebo (42.8% ± 4.8% area at risk; $P < 0.05$). This protection was reversed by co-administration of MPA with E (necrotic zone 38.2% ± 6.1% area at risk). The influence of E on neutrophil infiltration was demonstrated by its ability to reduce myocardial myeloperoxidase activity (0.2 ± 0.1 U/g tissue) relative to placebo (1.3 ± 0.5 U/g tissue; $P < 0.05$). Myocardial myeloperoxidase activity was significantly increased to 1.1 ± 0.3 U/g tissue in rats receiving E and MPA. However, MPA also reversed the protective effect of E in neutrophil-free buffer-perfused hearts, suggesting that additional mechanisms are involved.

Conclusion: In this study, we showed that the administration of MPA can inhibit the effects of E that lead to protection of the myocardium from reperfusion injury and that this involves both neutrophil-dependent and neutrophil-independent mechanisms.

Key Words: Estrogen – Medroxyprogesterone acetate – Myocardium – Ischemia.

Cardiovascular disease is the leading cause of mortality and morbidity in women.[1] Although the rate of cardiovascular disease is lower in premenopausal women than in age-matched men, it increases significantly postmenopause, reaching levels found among men. The observed gender difference and time of onset suggest that estrogen (E) withdrawal and, consequently, E deficiency, is an important risk factor. This is supported by observational studies showing postmenopausal estrogen therapy (ET) to be beneficial.[2,3] However, ET is only suitable for women who have undergone hysterectomy, as it can increase the risk of endometrial cancer. For this reason, most women receive E concurrently with a progestogen analog such as medroxyprogesterone acetate (MPA) in estrogen and progestogen therapy (EPT) to reduce this risk. The results of several recent large-scale clinical trials comparing ET with EPT have stimulated debate regarding the potential detrimental influence of progestogens in EPT.[4,5] Evidence is now emerging that MPA may interact with E at a cellular level, inhibiting its favorable effects.[6-8]

Received December 29, 2004; revised and accepted June 21, 2005.

*These authors jointly share first authorship.

[1]Centre for Cardiovascular Science, University of Edinburgh, Edinburgh, United Kingdom; [2]Department of Pharmacology, Mahidol University, Rajthevi, Bangkok, Thailand.

P.W. was funded by a Wellcome Trust travel award. H.J. was supported by the British Heart Foundation (FS/03114).

Address correspondence to: Helen Jeanes, E3, 24 Centre for Cardiovascular Science, University of Edinburgh, Queens Medical Research Institute, Little France, Edinburgh, EH16 4TJ Scotland. E-mail: h.l.jeanes@sms.ed.ac.uk.

In experimental models, E reduced myocardial injury er ischemia and reperfusion, at least in part as a result its ability to reduce neutrophil infiltration into the hemic myocardium.[9-11] The aim of the present study s to investigate whether the acute interactions reported ween MPA and E in vitro[7] could be reproduced after onic in vivo delivery, by investigating the influence MPA on the proven protective effect of E in ex-imental ischemia-reperfusion injury. Hearts from ariectomized rats chronically treated with hor-nes were studied both in vivo and in vitro to de-mine the relative importance of systemic and local eractions.

MATERIALS AND METHODS

This investigation conformed to the Guide for the re and Use of Laboratory Animals published by the ited States National Institutes of Health (NIH blication No 85-23, revised 1996) and with the ited Kingdom Home Office guidelines as outlined the Animals (Scientific Procedures) Act 1988. The dies were approved by the University of Edinburgh ical review committee. All animals were housed in ontrolled environment with a 12-hour light/dark cycle h constant temperature (21°C ± 2°C) and humidity, l they were fed a standard laboratory diet and tap ter ad libitum.

Female Wistar rats weighing 210 to 280 g (n = 61; arles Rivers Laboratories) were bilaterally ovariec-nized (halothane 4% induction, 1% to 1.2% mainte-nce). Briefly, the ovaries were exposed via a dorsal ision and tied off with a 5/0 silk ligature, then noved. The wound was closed using 5/0 silk ligature l 5-mm metal clips. The rats were then randomly signed to a treatment group, and sterile silicon pellets ntaining either E (3 mg; n = 14), MPA (3 mg; n = 13), + MPA (n = 15), or placebo (n = 19) were implanted bcutaneously. All rats underwent ovariectomy to sure that the endogenous supply of E was removed, l each treatment group only received supplemented rmones from the implanted pellets. Implanted pellets ve been used previously and were demonstrated to nieve a plasma level of E within physiological limits 500 pg/mL).[12] The animals were allowed to recover m anesthesia and were given buprenorphine hydrox-chloride (0.5 mg/kg) subcutaneously for analgesia. ts were weighed every 3 days until the end of the periment to ensure that they gained and maintained ight and to determine the influence of hormone plementation.

At least 14 days later, the rats were anesthetized (50 mg/kg sodium pentobarbitone intraperitoneally; Sagatal, Rhone Merieux, UK) and prepared for either in vivo or in vitro experiments.

In vivo

The carotid artery and jugular vein were cannulated to measure blood pressure and to deliver additional anesthetic as required. The rats were intubated and ventilated with room air (60 strokes/min, 0.1 mL/g). Temperature was maintained at 37°C via a thermostatically controlled under-blanket, and the chest was then opened at the fourth intercostal space. The heart was removed from the chest by applying gentle pressure to either side of the incision (with forceps), and a 5/0 silk ligature was placed around the left coronary artery (LCA). The heart was returned to the chest, and the animals were allowed a 20-minute stabilization period before ischemia was induced using a reversible snare. After 45 minutes of ischemia, the snare was released, and the myocardium was reperfused for 2 hours. At the end of this reperfusion period, the LCA was permanently occluded, and 2 mL of Evans blue (1% in saline) was infused via the jugular vein to determine the area at risk (AAR) of infarction. The heart was then cut in half transversely through the nonischemic area and stored at −70°C for later measurement of ischemic damage or myeloperoxidase (MPO) activity. The uterus was removed and weighed to confirm appropriate hormone delivery.

Myeloperoxidase assay

Neutrophil infiltration and accumulation in the myocardium was assessed using MPO activity as a measure.[13] In this study, the hearts were separated into the AAR and nonischemic area before storage at −70°C. At the time of analysis, the AAR was homogenized in buffer containing 20 mM sodium phosphate buffer (pH 4.7), 0.015 M EDTA, and 0.1 M sodium chloride, then centrifuged at 10,000 rpm for 15 minutes at 4°C. The supernatant was discarded and the pellet was resuspended. The samples were homogenized for a second time in sodium phosphate buffer (pH 5.4) containing 0.5% hexacyltrimethylammonium bromide. This was then taken through four cycles of freeze-thaw, a 10-second sonication, and 15-minute centrifugation at 10,000 rpm, 4°C. A portion of this supernatant (30 μL) was aliquoted into a 96-well plate and mixed with 200 μL 0.05 M citrate phosphate buffer (pH 5) containing o-dianisidine dihydrochloride and 0.0015% hydrogen peroxide. The absorbance was measured at a wavelength

of 405 nm during a 20-minute period. MPO activity was defined as the quantity of enzyme degrading 1 μmol of peroxide per minute at 25°C expressed in units per gram of weight.

In vitro

The chest was opened, and a 5/0 silk ligature was passed around the LCA before the heart was isolated. At this time, the uterus was also removed and weighed. The aorta was cannulated, and the heart was retrogradely perfused via the aorta using a modified Langendorff set up.[14] Prefiltered Krebs Henseleit solution [pH 7.4 containing (in mM] NaCl 118, KCl 4.7, $CaCl_2$ 3.0, $MgSO_4$ 1.2, KH_2PO_4 1.2, EDTA 0.5, $NaHCO_3$ 25 and glucose 11] was perfused through the heart at a constant rate of 10 mL/min and at a constant temperature of 37°C, gassed with 95% O_2/5% CO_2. After a stabilization period of 20 minutes, the LCA was reversibly occluded in the same way as in vivo; ie, 45 minutes of ischemia followed by 2 hours of reperfusion. The ligature was then retied, and 2 mL of Evans blue dye (1% in saline) was perfused via the injection arm to identify the AAR. The heart was then cut in half transversely through the nonischemic area and stored at −70°C for later analysis.

Measurement of infarct size

Myocardial damage and the impact of treatment were assessed using triphenyltetrazolium chloride-Evans blue technique[15] with some modifications. In brief, the left half of the heart was cut into 2- to 3-mm transverse slices from the apex to the base in 1% saline. The slices were then incubated at 37°C for 15 minutes in 2,3,5-triphenyltetrazolium chloride (1% weight/volume in normal saline). The color was fixed in 10% formalin for 10 minutes, and the sections were rinsed in 1% saline for 10 minutes. The heart was split into three distinct areas: the nonischemic area stained blue from the initial Evans blue perfusion and the two zones of the AAR, the pink non-necrotic and whiter necrotic zones. The distinct areas were separated, blotted dry, and weighed.

Statistical analysis

Statistical analysis was performed using PRISM software (GraphPad, CA). One-way analysis of variance and a Tukey post hoc test were applied to compare data. Statistical difference was taken as $P < 0.05$.

All chemicals and drugs were supplied by Sigma-Aldrich.

RESULTS

Body and uterine weight

There was no significant difference among the body weight of rats in the different treatment groups at the beginning of the study (Table 1). At the end of the study, ovariectomized rats treated with E weighed significantly less than animals receiving placebo or MPA alone ($P < 0.01$; Table 1). Co-administration of MPA failed to influence this effect of E.

E treatment also resulted in an increase in uterine weight relative to placebo ($P < 0.001$; Table 1). MPA had no effect on uterine weight when administered alone and had no influence on the ability of E to increase uterine weight.

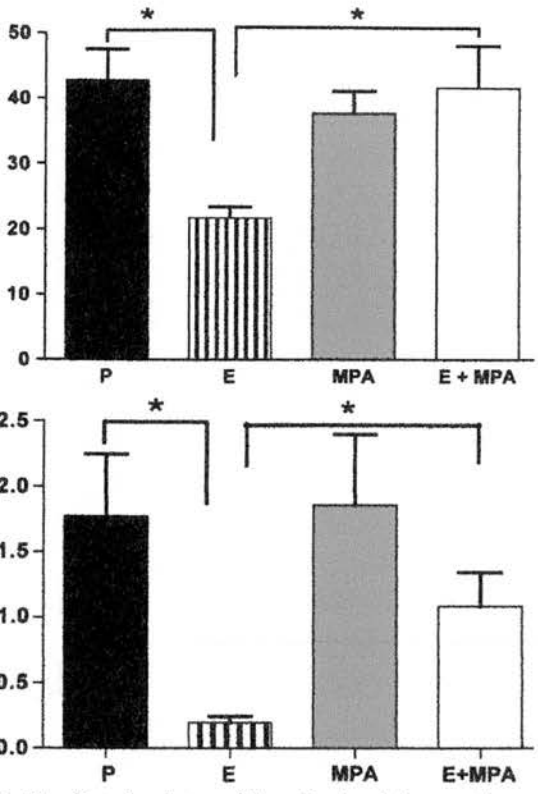
In vivo

No treatment significantly altered the size of the AAR (Table 1). Although there was no difference in the overall AAR, treatment with E alone significantly reduced the necrotic zone within it, compared with placebo treatment ($P < 0.05$; Fig. 1A). Treatment with MPA and E in combination resulted in a significantly ($P < 0.05$) larger necrotic zone compared with that in animals treated with E alone, such that there was no longer protection relative to placebo treatment (Fig. 1A). The size of the necrotic zone in rats receiving MPA alone was not significantly different from the necrotic zone in placebo-treated animals.

TABLE 1. *Effect of treatments on body weight and the area at risk after myocardial infarction*

Treatment	Initial body weight (g)[a]	Final body weight (g)[a]	Uterine weight (% of body weight)[a]	AAR % heart (g) (in vivo)	AAR % heart (g) (in vitro)
P	199.6 ± 6.7	272.8 ± 9.4	0.1 ± 0.01[c]	62.3 ± 6.8	70.4 ± 4.5
E	199.5 ± 5.2	231.0 ± 8.4[b]	0.3 ± 0.02	54.6 ± 3.6	67.3 ± 6.7
MPA	191.6 ± 8.5	254.4 ± 11.6	0.1 ± 0.01[c]	64.9 ± 6.1	63.8 ± 2.5
E + MPA	202.7 ± 6.7	229.8 ± 4.4[b]	0.3 ± 0.02	63.7 ± 5.0	70.8 ± 4.8

P, placebo; E, estrogen alone; MPA, medroxyprogesterone acetate alone; E + MPA, combination of hormone treatments; AAR, area at risk.
Data shown as mean ± SEM.
[a]Body weight and uterine weight, n = 14-19; AAR, n = 4-8.
[b]$P < 0.01$ compared with placebo.
[c]$P < 0.001$ compared with E-supplemented group.



1. The effect of ovariectomy followed by chronic hormone therapy
A) myocardial necrotic area after ischemia-reperfusion in vivo. The
tic zone is expressed as a percentage of the total area at risk (AAR)
B) myocardial myeloperoxidase (MPO) activity in the AAR of rats
cted to ischemia-reperfusion in vivo. Data are expressed as U/g
. Data shown as mean ± SEM. One-way analysis of variance with
y post hoc, $*P < 0.05$. n = 4-6 per group. P, placebo; E, estrogen;
, medroxyprogesterone acetate; E + MPA, estrogen and medroxy-
sterone acetate.

he mean blood pressure before ischemia was 71.8 ±
mm Hg and was not significantly different among
tment groups. There was a significant ($P < 0.05$)
ease in pressure at the onset of ischemia (to 40.5 ±
mm Hg) that increased within 30 minutes of ische-
to values not significantly different from those
re the onset of ischemia. None of the treatments
ienced the characteristic change in blood pressure
ischemia or reperfusion.

eloperoxidase assay

upplementation with E significantly ($P < 0.05$)
ced the amount of MPO activity in the AAR
pared with placebo (Fig. 1B). MPA supplementa-
alone did not significantly alter the MPO activity
pared with placebo treatment. However, when MPA and E were given in combination, the amount of MPO activity in the AAR was significantly increased compared with treatment with E alone ($P < 0.05$; Fig. 1B).

In vitro

We found a pattern similar to that seen in vivo. The size of the AAR in vitro was comparable to that in vivo; there was no significant difference among the AAR in any treatment group (Table 1). Figure 2 shows that treatment with E tended to reduce the necrotic zone compared with placebo, although this failed to reach significance ($P = 0.057$). Treatment with MPA alone did not significantly alter the necrotic zone relative to placebo. There was however, a significant ($P < 0.05$) increase in the necrotic area within the AAR in the group treated with E and MPA in combination, compared with treatment with E alone (Fig. 2).

All groups had a significant ($P < 0.05$) decrease in coronary perfusion pressure at the onset of ischemia, from 74.1 ± 4.6 mm Hg to 48.0 ± 3.2 mm Hg, which recovered during the first 30 minutes of ischemia. None of the treatments altered these characteristic changes in perfusion pressure throughout ischemia and reperfusion.

DISCUSSION

Large-scale prospective, double-blinded studies, such as the Women's Health Initiative,[16] Heart and Estrogen/progestin Replacement Study (HERS),[17] and Estrogen Replacement and Atherosclerosis (ERA) trial[18] have failed to demonstrate the cardiovascular benefits of EPT regimens in postmenopausal women.[19] Such

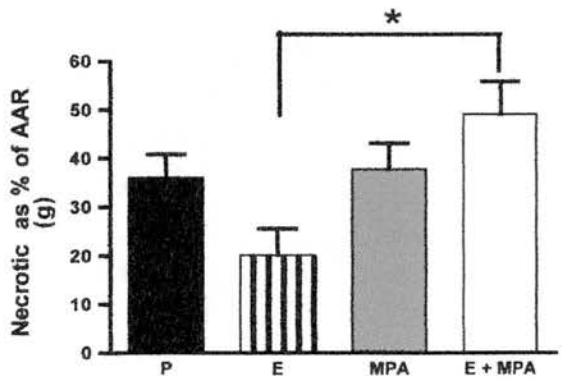


FIG. 2. Myocardial necrotic area after in vitro ischemia-reperfusion. The necrotic zone is expressed as a percentage of the total area at risk (AAR). P, placebo; E, estrogen; MPA, medroxyprogesterone acetate; E + MPA, estrogen and medroxyprogesterone acetate. Data shown as mean ± SEM. One-way analysis of variance with Tukey post-hoc $*P < 0.05$; n = 5-8.

findings were not expected and contradicted those obtained in observational studies of ET.[3,20,21] These discrepancies lead to a hypothesis that progestogens may inhibit the beneficial properties of E. This has led to a number of studies that have investigated potential interactions between E and progestogens.[6-8,22-25] Recently it was reported that MPA inhibited E-induced NO production when administered acutely to cultured cell lines.[6,7] The current findings clearly demonstrate that the ability of E to protect hearts from ischemic injury when chronically administered in vivo to ovariectomized rats is also inhibited by the co-administration of the progestogen MPA.

The protective effects of E in experimental models of myocardial ischemia-reperfusion[9-11] and vascular injury are well known.[26,27] In the present study, myocardial injury after ischemia and reperfusion was reduced in ovariectomized rats that had been treated with E compared with those receiving placebo. Damage to the myocardium in this model results from hypoxia-induced vasoconstriction and tissue necrosis[28,29] and from reperfusion-associated infiltration of activated inflammatory cells, increased oxidative stress, calcium overload, and reduced tissue adenosine triphosphate levels.[30-32] Several mechanisms have been proposed to account for the protective effects of E in this model.[33-36] Among these, stimulation of NO synthesis and activity may play a central role.[37] Increased NO bioavailability not only leads to increased vasodilation and reduction of hypoxia, but also contributes to the inhibition of inflammatory cell infiltration.[38] In the present study, reduction of reperfusion damage in vivo by E was associated with a reduction in tissue levels of MPO, indicative of a reduction in neutrophil infiltration, in agreement with previous observations.[9] However, this mechanism alone is clearly not sufficient to explain the protective effects of E found in this model. In hearts isolated from E-treated rats and perfused with neutrophil-free buffer in vitro, myocardial damage also tended to be reduced relative to that in hearts from placebo-treated rats, although to a lesser extent than in vivo. The antioxidant and vasodilator properties of E may have an important influence.[39]

Administration of the progestogen MPA in place of E had no influence on any of the parameters investigated in the present study. However, when co-administrated with E, MPA clearly inhibited E's protective effects in the heart. The necrotic zone of hearts from animals treated with E and MPA was comparable to that of placebo-treated animals, both in vivo and in vitro. In addition, in the in vivo experiments, MPO activity was almost reversed to placebo levels on co-administration of MPA with E, which indicates that inhibition of neutrophil infiltration by E was impaired. Interestingly, MPA did not prevent all systemic effects of E. Although body weight was characteristically reduced in E-treated rats,[40] MPA had no influence on body weight either alone or in combination with E. Similarly, MPA had no influence on uterine weight. E stimulated the proliferation of uterine epithelial cells[41] in both the absence and presence of MPA. MPA has previously been reported to act systemically and to prevent these effects of E.[22,42] However, in the present study, the level of MPA administered in this study seemed sufficient only to inhibit E in the heart, which suggests that a more sensitive mechanism is involved.

Several mechanisms have been proposed to account for the interactions between progestogens and other steroid hormones. The androgenic progestogen MPA was recently shown to impair E receptor signaling in endothelial cells,[6] leading to a reduction in its ability to increase NO bioavailability.[7] MPA also has antagonistic properties at glucocorticoid receptors, leading to an increased ability of human leukocytes to express the adhesion molecule ICAM-1.[6] The binding of E to its receptors is also reported to be inhibited by MPA,[43] and progestogens like MPA are reported to down-regulate the E receptor in target tissues.[42,44] Complete inhibition of E binding to its receptors seems an unlikely explanation for the observations in the present study, as this would also have limited the effects of E on the body weight and uterine proliferation, unless E receptors in cardiovascular target tissue are down-regulated at a lower concentration of MPA than that required in other target tissues. Impaired receptor signaling and interaction with other steroid receptors that may cooperate with E are attractive alternative hypotheses. A reduction by MPA on the ability of E to increase NO bioavailability would lead to impairment of the vasodilatory and anti-inflammatory effects of E in this ischemia reperfusion injury model. Indeed, progestogens have previously been shown to interfere with E-induced enhancement of coronary flow[23] and endothelium-dependant dilatation.[45,46] Inhibition of neutrophil infiltration would also be enhanced by MPA via reduced NO availability and by increased expression of adhesion molecules like ICAM-1.

CONCLUSION

It is clear from this study that, when co-administered in vivo with E, MPA has the ability to negate the protective effects of E in the myocardium. These data further suggest that interactions between E and progestogens such as MPA can lead to inhibition of E's

-inflammatory effects and enhanced inflammatory accumulation, as well as enhancement of other ergistic mechanisms that protect the tissue more ectly from ischemic injury.

REFERENCES

Pelzer T, Shamim A, Neyses L. Estrogen effects in the heart. *Mol Cell Biochem* 1996;160-161:307-313.

Grodstein F, Manson JE, Colditz GA, Willett WC, Speizer FE, Stampfer MJ. A prospective, observational study of postmenopausal hormone therapy and primary prevention of cardiovascular disease. *Ann Intern Med* 2000;133:933-941.

Bush TL, Barrett-Connor E, Cowan LD, et al. Cardiovascular mortality and noncontraceptive use of estrogen in women: results from the Lipid Research Clinics Program Follow-up Study. *Circulation* 1987;75:1102-1109.

Manson JE, Hsia J, Johnson KC, et al. Estrogen plus progestin and the risk of coronary heart disease. *N Engl J Med* 2003;349:523-534.

Dubey RK, Imthurn B, Zacharia LC, Jackson EK. Hormone replacement therapy and cardiovascular disease: what went wrong and where do we go from here? *Hypertension* 2004;44:789-795.

Simoncini T, Mannella P, Fornari L, et al. Differential signal transduction of progesterone and medroxyprogesterone acetate in human endothelial cells. *Endocrinology* 2004;145:5745-5756.

Oishi A, Ohmichi M, Takahashi K, et al. Medroxyprogesterone acetate attenuates estrogen-induced nitric oxide production in human umbilical vein endothelial cells. *Biochem Biophys Res Commun* 2004;324:193-198.

Koh KK, Sakuma I. Should progestins be blamed for the failure of hormone replacement therapy to reduce cardiovascular events in randomized controlled trials? *Arterioscler Thromb Vasc Biol* 2004;24:1171-1179.

Squadrito F, Altavilla D, Squadrito G, et al. 17Beta-oestradiol reduces cardiac leukocyte accumulation in myocardial ischaemia reperfusion injury in rat. *Eur J Pharmacol* 1997;335:185-192.

Kolodgie FD, Farb A, Litovsky SH, et al. Myocardial protection of contractile function after global ischemia by physiologic estrogen replacement in the ovariectomized rat. *J Mol Cell Cardiol* 1997; 29:2403-2414.

Zhai P, Eurell TE, Cotthaus R, Jeffery EH, Bahr JM, Gross DR. Effect of estrogen on global myocardial ischemia-reperfusion injury in female rats. *Am J Physiol Heart Circ Physiol* 2000;279:H2766-H2775.

Fink G, Sumner BE, McQueen JK, Wilson H, Rosie R. Sex steroid control of mood, mental state and memory. *Clin Exp Pharmacol Physiol* 1998;25:764-775.

Mullane KM, Kraemer R, Smith B. Myeloperoxidase activity as a quantitative assessment of neutrophil infiltration into ischemic myocardium. *J Pharmacol Methods* 1985;14:157-167.

Rubanyi G, Kovach AG. Effect of hypoxia on mechanical properties of isolated rat heart: comparison of the effects of hypoxic and histotoxic hypoxia. *Acta Physiol Acad Sci Hung* 1980;55:215-225.

de Lorgeril M, Rousseau G, Basmadjian A, Latour JG. Lignocaine in experimental myocardial infarction: failure to prevent neutrophil accumulation and ventricular fibrillation and to reduce infarct size. *Cardiovasc Res* 1988;22:439-446.

The Women's Health Initiative Study Group. Design of the Women's Health Initiative clinical trial and observational study. *Control Clin Trials* 1998:19:61-109.

Hulley S, Grady D, Bush T. Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women: Heart and Estrogen/progestin Replacement Study (HERS) Research Group. *JAMA* 1998;280:605-613.

Herrington DM, Reboussin DM, Brosnihan KB. Effects of estrogen replacement on the progression of coronary-artery atherosclerosis. *N Engl J Med* 2000;343:522-529.

Langer RD. Hormone replacement and the prevention of cardiovascular disease. *Am J Cardiol* 2002;89:36E–46E [discussion 46E].

Henderson BE, Paganini-Hill A, Ross RK. Decreased mortality in users of estrogen replacement therapy. *Arch Intern Med* 1991;151: 75-78.

Falkeborn M, Persson I, Adami HO. The risk of acute myocardial infarction after oestrogen and oestrogen-progestogen replacement. *Br J Obstet Gynaecol* 1992;99:821-828.

McNeill AM, Zhang C, Stanczyk FZ, Duckles SP, Krause DN. Estrogen increases endothelial nitric oxide synthase via estrogen receptors in rat cerebral blood vessels: effect preserved after concurrent treatment with medroxyprogesterone acetate or progesterone. *Stroke* 2002;33:1685-1691.

Gorodeski GI, Yang T, Levy MN, Goldfarb J, Utian WH. Modulation of coronary vascular resistance in female rabbits by estrogen and progesterone. *J Soc Gynecol Invest* 1998;5:197-202.

Minshall RD, Stanczyk FZ, Miyagawa K, et al. Ovarian steroid protection against coronary artery hyperreactivity in rhesus monkeys. *J Clin Endocrinol Metab* 1998;83:649-659.

Adams MR, Register TC, Golden DL, Wagner JD, Williams JK. Medroxyprogesterone acetate antagonizes inhibitory effects of conjugated equine estrogens on coronary artery atherosclerosis. *Arterioscler Thromb Vasc Biol* 1997;17:217-221.

Sullivan TR Jr, Karas RH, Aronovitz M, et al. Estrogen inhibits the response-to-injury in a mouse carotid artery model. *J Clin Invest* 1995;96:2482-2488.

Levine RL, Chen SJ, Durand J, Chen YF, Oparil S. Medroxyprogesterone attenuates estrogen-mediated inhibition of neointima formation after balloon injury of the rat carotid artery. *Circulation* 1996;94:2221-2227.

Lefer AM. Physiologic and pathophysiologic role of cyclooxygenase metabolites of arachidonic acid in circulatory disease states. *Cardiovasc Clin* 1987;18:85-99.

Jennings RB, Reimer KA. Factors involved in salvaging ischemic myocardium: effect of reperfusion of arterial blood. *Circulation* 1983;68(2 Pt 2):I25–I36.

McCord JM. Oxygen-derived free radicals in postischemic tissue injury. *N Engl J Med* 1985;312:159-163.

Premaratne S, Limm W, Mugiishi MM, Piette LH, McNamara JJ. Quantitative assessment of free-radical generation during ischemia and reperfusion in the isolated rabbit heart. *Coronary Artery Dis* 1993;4:1093-1096.

Hudson KF. A phenomenon of paradox: myocardial reperfusion injury. *Heart Lung* 1994;23:384-393.

Meade TW, Berra A. Hormone replacement therapy and cardiovascular disease. *Br Med Bull* 1992;48:276-308.

Wahl P, Walden C, Knopp R, et al. Effect of estrogen/progestin potency on lipid/lipoprotein cholesterol. *N Engl J Med* 1983;308: 862-867.

Lucchesi BR. Modulation of leukocyte-mediated myocardial reperfusion injury. *Ann Rev Physiol* 1990;52:561-576.

Stampfer MJ, Colditz GA. Estrogen replacement therapy and coronary heart disease: a quantitative assessment of the epidemiologic evidence. *Prev Med* 1991;20:47-63.

Rubanyi GM, Freay AD, Kauser K, et al. Vascular estrogen receptors and endothelium-derived nitric oxide production in the mouse aorta: gender difference and effect of estrogen receptor gene disruption. *J Clin Invest* 1997;99:2429-2437.

Davies MG, Fulton GJ, Hagen PO. Clinical biology of nitric oxide. *Br J Surg* 1995;82:1598-1610.

Babiker FA, De Windt LJ, van Eickels M, Grohe C, Meyer R, Doevendans PA. Estrogenic hormone action in the heart: regulatory network and function. *Cardiovasc Res* 2002;53:709-719.

Beckett T, Tchernof A, Toth MJ. Effect of ovariectomy and estradiol replacement on skeletal muscle enzyme activity in female rats. *Metabolism* 2002;51:1397-1401.

41. Bigsby RM, Caperell-Grant A, Berry N, Nephew K, Lubahn D. Estrogen induces a systemic growth factor through an estrogen receptor-alpha-dependent mechanism. *Biol Reprod* 2004;70:178-183.
42. Di Carlo F, Racca S, Conti G, et al. Effects of long-term administration of high doses of medroxyprogesterone acetate on hormone receptors and target organs in the female rat. *J Endocrinol* 1984;103:287-293.
43. Di Carlo F, Gallo E, Conti G, Racca S. Changes in the binding of oestradiol to uterine oestrogen receptors induced by some progesterone and 19-nor-testosterone derivatives. *J Endocrinol* 1983;98:385-389.
44. Chwalisz K, Stockemann K, Fritzemeier KH, Fuhrmann U. Modulation of oestrogenic effects by progesterone antagonists in the rat uterus. *Hum Reprod Update* 1998;4:570-583.
45. Chataigneau T, Zerr M, Chataigneau M, et al. Chronic treatment with progesterone but not medroxyprogesterone acetate restores the endothelial control of vascular tone in the mesenteric artery of ovariectomized rats. *Menopause* 2004;11:255-263.
46. Wakatsuki A, Okatani Y, Ikenoue N, Fukaya T. Effect of medroxyprogesterone acetate on endothelium-dependent vasodilation in postmenopausal women receiving estrogen. *Circulation* 2001;104:1773-1778.

MEDROXYPROGESTERONE ACETATE INHIBITS THE CARDIOPROTECTIVE EFFECT OF OESTROGEN AFTER ISCHAEMIA-REPERFUSION IN THE RAT.

Helen Jeanes, Isam Sharif, Payong Wanikiat & Gillian A Gray, Centre for Cardiovascular Science, The University of Edinburgh, Hugh Robson Building, George Square, Edinburgh, EH8 9LD, UK.

Cardioprotection by oestrogen (E) is clear from epidemiological studies in pre- and post-menopausal women, as well as in animal models. However recent clinical trials, such as the Women's Health Initiative (WHI) and the Heart and Estrogen/Progestin Replacement Study (HERS), have demonstrated that hormone replacement therapy (HRT) which contains a mixture of both E and medroxyprogesterone acetate (MPA), does not protect postmenopausal women from cardiovascular disease and could actually increase the incidence rate (Herrington *et al.*, 2003).

The aim of the current study was to investigate whether MPA can modify the cardioprotective effect of E in a rat model of ischaemia-reperfusion.

Female rats (Wistar 150-200g, n=45) underwent ovariectomy (OVX). Pellets containing E alone, MPA alone, E + MPA or placebo were then implanted subcutaneously. At least 14 days later, hearts were removed for *in vitro* perfusion or rats were prepared for *in vivo* studies. In both cases the left coronary artery (LCA) was reversibly occluded for 45 minutes and then reperfused for 2 hours. The LCA was re-occluded and Evans blue perfused through the heart for identification of the area-at-risk (AAR). Myocardial damage assessed in sections of frozen heart using triphenyl tetrazolium chloride.

AAR after reperfusion was not significantly different between any treatment group, being 68 $\pm$ 1.5 % *in vitro* and 61 $\pm$ 2 % *in vivo*. E reduced the necrotic zone both *in vivo*, (see Figure 1) and *in vitro*, from 45 $\pm$ 5% of AAR to 25 $\pm$ 2% of AAR. MPA itself failed to modify the infarct size. However, when given together MPA inhibited the

cardioprotective effect of E, both in vivo (see Figure 1) and *in vitro* (necrotic zone 52 ± 6%, P<0.05 versus E alone).

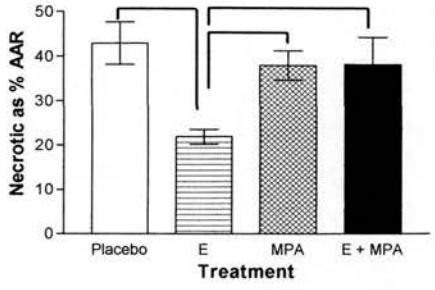


Figure 1. Ischaemic damage *in vivo*.
(n=5, 4 for MPA. $^{**}p<0.01$, $^{*}p<0.05$
Tukey following 1-way ANOVA) .

These confirm cardioprotection by E, and show that protection is negated on co-administration of MPA, both *in vivo* and *in vitro* buffered perfused hearts. This maybe due to altered neutrophil infiltration *in vivo* but other mechanisms e.g. antioxidant effects may also contribute *in vitro*. MPA may act through modification of E receptor synthesis or of E binding to them.

Herrington, D.M. *et al.* (2003) Atherosclerosis, 166, 203-212

This research was funded by the British Heart Foundation (FS03114).

17β-ESTRADIOL MODIFIES THE PROFILE OF MYOCARDIAL MMP AND TIMP DURING HEALING POST-MYOCARDIAL INFARCTION IN MICE

H.L.Jeanes[1,2], I.Sharif[1], A.Kozak[1], R.Leask[2], E.Bruce[1,2], S.C.Riley[2] & G.A.Gray[1].
[1]CCVS & [2]CRB, University of Edinburgh, QMRI, Edinburgh, UK

Pre-menopausal women have significantly greater acute mortality post-myocardial infarction (MI) than age-matched men. Ovariectomised (OVX) mice receiving 17β-estradiol (E_2) have significantly greater mortality post-MI due to cardiac rupture compared to placebo (P) treated mice. Matrix metalloproteinases (MMP) regulate turnover of the extracellular matrix (ECM), MMP activity is regulated by the tissue inhibitors of metalloproteinases (TIMPs). E_2 regulates activity and expression of MMP in a number of tissues, it's effects in the myocardium are unknown.

The aim of the present study was to determine the impact of E_2 on infarct size, neutrophil infiltration, expression and secreted activity of the gelatinases MMP-2 and –9, the collagenase MMP-13 and the TIMPs during infarct healing post-MI.

OVX female C57B6J/129sv (n=34) had E_2 or P releasing pellets implanted subcutaneously and eight days later MI or sham surgery. Neutrophil infiltration and infarct size were determined at 2 days post-MI by immunohistochemistry and triphenyltetrazolium chloride respectively. Secreted activity of MMP-2 and –9 were assayed by gelatin zymography, TIMP activity was assayed by reverse zymography, expression of TIMP-2 and MMP-13 was determined by western blotting in tissue removed at 4 days post-MI.

MI significantly ($P<0.001$) increased neutrophil infiltration, the secreted activity of MMP-2 and MMP-9 and MMP-13 expression in the infarct ($P<0.05$). E_2 reduced both infarct size and neutrophil infiltration, but had no influence on gelatinase activity. E did reduce the expression of MMP-13 and increased TIMP-2 activity.

These results show E_2 increases sudden death due to cardiac rupture despite reducing damage to the myocardium post-MI and subsequent neutrophil infiltration. The continued stimulation of MMP-2 and -9 activity in E_2 treated hearts may contribute to destabilisation of the healing infarct, resulting in deficient repair and rupture.

Adams, M. R., T. C. Register, et al. (1997). "Medroxyprogesterone acetate antagonizes inhibitory effects of conjugated equine estrogens on coronary artery atherosclerosis." *Arterioscler Thromb Vasc Biol* **17**(1): 217-21.

Alexander, J. P., J. R. Samples, et al. (1998). "Growth factor and cytokine modulation of trabecular meshwork matrix metalloproteinase and TIMP expression." *Curr Eye Res* **17**(3): 276-85.

Allan, J. A., A. J. Docherty, et al. (1995). "Binding of gelatinases A and B to type-I collagen and other matrix components." *Biochem J* **309 (Pt 1)**: 299-306.

Alon, R., T. Feizi, et al. (1995). "Glycolipid ligands for selectins support leukocyte tethering and rolling under physiologic flow conditions." *J Immunol* **154**(10): 5356-66.

Amant, C. and P. Holm (2001). "Estrogen receptor-mediated, nitric oxide dependent modulation of the immunologic barrier function of the endothelium: regulation of fas ligand expression by estradiol " *Circulation* **104**(21): 2576-81.

Anderson, G. L., M. Limacher, et al. (2004). "Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the Women's Health Initiative randomized controlled trial." *Jama* **291**(14): 1701-12.

Ariazi, E. A. and V. C. Jordan (2006). "Estrogen-related receptors as emerging targets in cancer and metabolic disorders." *Curr Top Med Chem* **6**(3): 203-15.

Arras, M., W. D. Ito, et al. (1998). "Monocyte activation in angiogenesis and collateral growth in the rabbit hindlimb." *J Clin Invest* **101**(1): 40-50.

Ashcroft, G. S., T. Greenwell-Wild, et al. (1999). "Topical estrogen accelerates cutaneous wound healing in aged humans associated with an altered inflammatory response." *Am J Pathol* **155**(4): 1137-46.

Assoian, R. K. and E. E. Marcantonio (1997). "The extracellular matrix as a cell cycle control element in atherosclerosis and restenosis." *J Clin Invest* **100**(11 Suppl): S15-8.

Babiker, F. A., L. J. De Windt, et al. (2002). "Estrogenic hormone action in the heart: regulatory network and function." *Cardiovasc Res* **53**(3): 709-19.

Baker, A. H., D. R. Edwards, et al. (2002). "Metalloproteinase inhibitors: biological actions and therapeutic opportunities." *J Cell Sci* **115**(Pt 19): 3719-27.

Balestrini, J. L. and K. L. Billiar (2006). "Equibiaxial cyclic stretch stimulates fibroblasts to rapidly remodel fibrin." *J Biomech* **39**(16): 2983-90.

Barbato, J. E. and E. Tzeng (2004). "Nitric oxide and arterial disease." *J Vasc Surg* **40**(1): 187-93.

Barp, J., A. S. Araujo, et al. (2002). "Myocardial antioxidant and oxidative stress changes due to sex hormones." *Braz J Med Biol Res* **35**(9): 1075-81.

Barrett-Connor, E., L. Mosca, et al. (2006). "Effects of raloxifene on cardiovascular events and breast cancer in postmenopausal women." *N Engl J Med* **355**(2): 125-37.

Baudino, T. A., W. Carver, et al. (2006). "Cardiac fibroblasts: friend or foe?" *Am J Physiol Heart Circ Physiol* **291**(3): H1015-26.

Becker, J. W., A. I. Marcy, et al. (1995). "Stromelysin-1: three-dimensional structure of the inhibited catalytic domain and of the C-truncated proenzyme." *Protein Sci* **4**(10): 1966-76.

Becker, R. C., J. M. Gore, et al. (1996). "A composite view of cardiac rupture in the United States National Registry of Myocardial Infarction." *J Am Coll Cardiol* **27**(6): 1321-6.

Beckett, T., A. Tchernof, et al. (2002). "Effect of ovariectomy and estradiol replacement on skeletal muscle enzyme activity in female rats." *Metabolism* **51**(11): 1397-401.

Beer, S., M. Reincke, et al. (2002). "Susceptibility to cardiac ischemia/reperfusion injury is modulated by chronic estrogen status." *J Cardiovasc Pharmacol* **40**(3): 420-8.

Beer, S., M. Reincke, et al. (2006). "High-dose 17beta-estradiol treatment prevents development of heart failure post-myocardial infarction in the rat." *Basic Res Cardiol.*

Bigsby, R. M., A. Caperell-Grant, et al. (2004). "Estrogen induces a systemic growth factor through an estrogen receptor-alpha-dependent mechanism." *Biol Reprod* **70**(1): 178-83.

Birdsall, H. H., D. M. Green, et al. (1997). "Complement C5a, TGF-beta 1, and MCP-1, in sequence, induce migration of monocytes into ischemic canine myocardium within the first one to five hours after reperfusion." *Circulation* **95**(3): 684-92.

Biswas, C., Y. Zhang, et al. (1995). "The human tumor cell-derived collagenase stimulatory factor (renamed EMMPRIN) is a member of the immunoglobulin superfamily." *Cancer Res* **55**(2): 434-9.

Blankesteijn, W. M., E. Creemers, et al. (2001). "Dynamics of cardiac wound healing following myocardial infarction: observations in genetically altered mice." *Acta Physiol Scand* **173**(1): 75-82.

Blumel, J. E., C. Castelo-Branco, et al. (2003). "Effects of transdermal estrogens on endothelial function in postmenopausal women with coronary disease." *Climacteric* **6**(1): 38-44.

Blumenthal, R. S., B. Baranowski, et al. (2004). "Cardiovascular effects of raloxifene: the arterial and venous systems." *Am Heart J* **147**(5): 783-9.

Bodden, M. K., G. J. Harber, et al. (1994). "Functional domains of human TIMP-1 (tissue inhibitor of metalloproteinases)." *J Biol Chem* **269**(29): 18943-52.

Bode, W. (1995). "A helping hand for collagenases: the haemopexin-like domain." *Structure* **3**(6): 527-30.

Bolli, R. (1991). "Oxygen-derived free radicals and myocardial reperfusion injury: an overview." *Cardiovasc Drugs Ther* **5 Suppl 2**: 249-68.

Booth, E. A., M. Marchesi, et al. (2003). "17Beta-estradiol as a receptor-mediated cardioprotective agent." *J Pharmacol Exp Ther* **307**(1): 395-401.

Booth, E. A., N. R. Obeid, et al. (2005). "Activation of estrogen receptor-alpha protects the in vivo rabbit heart from ischemia-reperfusion injury." *Am J Physiol Heart Circ Physiol* **289**(5): H2039-47.

Borras, C., J. Gambini, et al. (2005). "17beta-oestradiol up-regulates longevity-related, antioxidant enzyme expression via the ERK1 and ERK2[MAPK]/NFkappaB cascade." *Aging Cell* **4**(3): 113-8.

Brasselet, C., E. Durand, et al. (2005). "Collagen and elastin cross-linking: a mechanism of constrictive remodeling after arterial injury." *Am J Physiol Heart Circ Physiol* **289**(5): H2228-33.

Brown, R. D., S. K. Ambler, et al. (2005). "The cardiac fibroblast: therapeutic target in myocardial remodeling and failure." *Annu Rev Pharmacol Toxicol* **45**: 657-87.

Brzozowski, A. M., A. C. Pike, et al. (1997). "Molecular basis of agonism and antagonism in the oestrogen receptor." *Nature* **389**(6652): 753-8.

Buck, C. A., H. S. Baldwin, et al. (1993). "Cell adhesion receptors and early mammalian heart development: an overview." *C R Acad Sci III* **316**(9): 838-59.

Burke, A. P., A. Farb, et al. (1998). "Effect of risk factors on the mechanism of acute thrombosis and sudden coronary death in women." *Circulation* **97**(21): 2110-6.

Burlew, B. S. and K. T. Weber (2000). "Connective tissue and the heart. Functional significance and regulatory mechanisms." *Cardiol Clin* **18**(3): 435-42.

Bush, T. L., E. Barrett-Connor, et al. (1987). "Cardiovascular mortality and noncontraceptive use of estrogen in women: results from the Lipid Research Clinics Program Follow-up Study." *Circulation* **75**(6): 1102-9.

Butler, G. S., H. Will, et al. (1997). "Membrane-type-2 matrix metalloproteinase can initiate the processing of progelatinase A and is regulated by the tissue inhibitors of metalloproteinases." *Eur J Biochem* **244**(2): 653-7.

Butler, G. S., M. J. Butler, et al. (1998). "The TIMP2 membrane type 1 metalloproteinase "receptor" regulates the concentration and efficient activation of progelatinase A. A kinetic study." *J Biol Chem* **273**(2): 871-80.

Califf, R. M. (1998). "Ten years of benefit from a one-hour intervention." *Circulation* **98**(24): 2649-51.

Carmeliet, P. (2000). "Mechanisms of angiogenesis and arteriogenesis." *Nat Med* **6**(4): 389-95.

Cavasin, M. A., S. S. Sankey, et al. (2003). "Estrogen and testosterone have opposing effects on chronic cardiac remodeling and function in mice with myocardial infarction." *Am J Physiol Heart Circ Physiol* **284**(5): H1560-9.

Cavasin, M. A., Z. Tao, et al. (2004). "Gender differences in cardiac function during early remodeling after acute myocardial infarction in mice." *Life Sci* **75**(18): 2181-92.

Cavasin, M. A., Z. Y. Tao, et al. (2006). "Testosterone enhances early cardiac remodeling after myocardial infarction, causing rupture and degrading cardiac function." *Am J Physiol Heart Circ Physiol* **290**(5): H2043-50.

Chambliss, K. L., I. S. Yuhanna, et al. (2000). "Estrogen receptor alpha and endothelial nitric oxide synthase are organized into a functional signaling module in caveolae." *Circ Res* **87**(11): E44-52.

Chandra, N. C., R. C. Ziegelstein, et al. (1998). "Observations of the treatment of women in the United States with myocardial infarction: a report from the National Registry of Myocardial Infarction-I." *Arch Intern Med* **158**(9): 981-8.

Chataigneau, T., M. Zerr, et al. (2004). "Chronic treatment with progesterone but not medroxyprogesterone acetate restores the endothelial control of vascular tone in the mesenteric artery of ovariectomized rats." *Menopause* **11**(3): 255-63.

Chen, S. J., H. Li, et al. (1996). "Estrogen reduces myointimal proliferation after balloon injury of rat carotid artery." *Circulation* **93**(3): 577-84.

Chen, Z., I. S. Yuhanna, et al. (1999). "Estrogen receptor alpha mediates the nongenomic activation of endothelial nitric oxide synthase by estrogen." *J Clin Invest* **103**(3): 401-6.

Chwalisz, K., K. Stockemann, et al. (1998). "Modulation of oestrogenic effects by progesterone antagonists in the rat uterus." *Hum Reprod Update* **4**(5): 570-83.

Clark, C., M. I. Foreman, et al. (1980). "Coronary artery ligation in anesthetized rats as a method for the production of experimental dysrhythmias and for the determination of infarct size." *J Pharmacol Methods* **3**(4): 357-68.

Cleutjens, J. P. and E. E. Creemers (2002). "Integration of concepts: cardiac extracellular matrix remodeling after myocardial infarction." *J Card Fail* **8**(6 Suppl): S344-8.

Cleutjens, J. P., J. C. Kandala, et al. (1995b). "Regulation of collagen degradation in the rat myocardium after infarction." *J Mol Cell Cardiol* **27**(6): 1281-92.

Cleutjens, J. P., M. J. Verluyten, et al. (1995a). "Collagen remodeling after myocardial infarction in the rat heart." *Am J Pathol* **147**(2): 325-38.

Cohen, B. J., D. Danon, et al. (1987). "Wound repair in mice as influenced by age and antimacrophage serum." *J Gerontol* **42**(3): 295-301.

Corrado, D., C. Basso, et al. (1994). "Sudden death in the young. Is acute coronary thrombosis the major precipitating factor?" *Circulation* **90**(5): 2315-23.

Couse, J. F., S. W. Curtis, et al. (1995). "Analysis of transcription and estrogen insensitivity in the female mouse after targeted disruption of the estrogen receptor gene." *Mol Endocrinol* **9**(11): 1441-54.

Creemers, E. E., J. P. Cleutjens, et al. (2001). "Matrix metalloproteinase inhibition after myocardial infarction: a new approach to prevent heart failure?" *Circ Res* **89**(3): 201-10.

Creemers, E., J. Cleutjens, et al. (2000). "Disruption of the plasminogen gene in mice abolishes wound healing after myocardial infarction." *Am J Pathol* **156**(6): 1865-73.

Cunningham, F. M., M. J. Smith, et al. (1979). "Migration of peritoneal polymorphonuclear leucocytes in the rat." *J Pathol* **128**(1): 15-20.

Curran, S. and G. I. Murray (1999). "Matrix metalloproteinases in tumour invasion and metastasis." *J Pathol* **189**(3): 300-8.

Dalrymple-Hay, M. J., J. L. Monro, et al. (1998). "Postinfarction ventricular septal rupture: the Wessex experience." *Semin Thorac Cardiovasc Surg* **10**(2): 111-6.

Dangerfield, J., K. Y. Larbi, et al. (2002). "PECAM-1 (CD31) homophilic interaction up-regulates alpha6beta1 on transmigrated neutrophils in vivo and plays a functional role in the ability of alpha6 integrins to mediate leukocyte migration through the perivascular basement membrane." *J Exp Med* **196**(9): 1201-11.

Dano, K., P. A. Andreasen, et al. (1985). "Plasminogen activators, tissue degradation, and cancer." *Adv Cancer Res* **44**: 139-266.

Darley-Usmar, V. M., J. McAndrew, et al. (1997). "Nitric oxide, free radicals and cell signalling in cardiovascular disease." *Biochem Soc Trans* **25**(3): 925-9.

Davies, M. G., G. J. Fulton, et al. (1995). "Clinical biology of nitric oxide." *Br J Surg* **82**(12): 1598-610.

de Gooyer, M. E., G. H. Deckers, et al. (2003). "Receptor profiling and endocrine interactions of tibolone." *Steroids* **68**(1): 21-30.

Dellborg, M., P. Held, et al. (1985). "Rupture of the myocardium. Occurrence and risk factors." *Br Heart J* **54**(1): 11-6.

Delyani, J. A., T. Murohara, et al. (1996). "Protection from myocardial reperfusion injury by acute administration of 17 beta-estradiol." *J Mol Cell Cardiol* **28**(5): 1001-8.

Di Carlo, F., E. Gallo, et al. (1983). "Changes in the binding of oestradiol to uterine oestrogen receptors induced by some progesterone and 19-nor-testosterone derivatives." *J Endocrinol* **98**(3): 385-9.

Di Carlo, F., S. Racca, et al. (1984). "Effects of long-term administration of high doses of medroxyprogesterone acetate on hormone receptors and target organs in the female rat." *J Endocrinol* **103**(3): 287-93.

Dubey, R. K., B. Imthurn, et al. (2004). "Hormone replacement therapy and cardiovascular disease: what went wrong and where do we go from here?" *Hypertension* **44**(6): 789-95.

Dubey, R. K., E. K. Jackson, et al. (2001). "Estradiol metabolites inhibit endothelin synthesis by an estrogen receptor-independent mechanism." *Hypertension* **37**(2 Part 2): 640-4.

Dubey, R. K., S. P. Tofovic, et al. (2004). "Cardiovascular pharmacology of estradiol metabolites." *J Pharmacol Exp Ther* **308**(2): 403-9.

Ducharme, A., S. Frantz, et al. (2000). "Targeted deletion of matrix metalloproteinase-9 attenuates left ventricular enlargement and collagen accumulation after experimental myocardial infarction." *J Clin Invest* **106**(1): 55-62.

Duncan, G. S., D. P. Andrew, et al. (1999). "Genetic evidence for functional redundancy of Platelet/Endothelial cell adhesion molecule-1 (PECAM-1): CD31-deficient mice reveal PECAM-1-dependent and PECAM-1-independent functions." *J Immunol* **162**(5): 3022-30.

Ederveen, A. G. and H. J. Kloosterboer (1999). "Tibolone, a steroid with a tissue-specific hormonal profile, completely prevents ovariectomy-induced bone loss in sexually mature rats." *J Bone Miner Res* **14**(11): 1963-70.

Ederveen, A. G. and H. J. Kloosterboer (2001). "Tibolone exerts its protective effect on trabecular bone loss through the estrogen receptor." *J Bone Miner Res* **16**(9): 1651-7.

Eghbali, M., M. J. Czaja, et al. (1988). "Collagen chain mRNAs in isolated heart cells from young and adult rats." *J Mol Cell Cardiol* **20**(3): 267-76.

ELITE. (2005). "ELITE trial." from http://www.clinicaltrials.gov/show/NCT00114517.

Engler, R. L., G. W. Schmid-Schanbein, et al. (1983). "Leukocyte capillary plugging in myocardial infarction and reperfusion in dogs." *Am J Pathol* **111**: 98-111.

Entman, M. L. and C. W. Smith (1994). "Postreperfusion inflammation: a model for reaction to injury in cardiovascular disease." *Cardiovasc Res* **28**(9): 1301-11.

Erlandsson, M. C., U. Islander, et al. (2005). "Estrogenic agonism and antagonism of the soy isoflavone genistein in uterus, bone and lymphopoiesis in mice." *Apmis* **113**(5): 317-23.

ESHRE Capri workshop group (2006). "Hormones and cardiovascular health in women." *Human reproduction update* **12**(5): 483-497.

Evans, M. J. and A. Eckert (2001). "Reciprocal antagonism between estrogen receptor and NF-kappa B activity in vivo." *Circ Res* **89**(9): 823-30.

Falkeborn, M., I. Persson, et al. (1992). "The risk of acute myocardial infarction after oestrogen and oestrogen-progestogen replacement." *Br J Obstet Gynaecol* **99**(10): 821-8.

Fedak, P. W., S. Verma, et al. (2005). "Cardiac remodeling and failure From molecules to man (Part II)." *Cardiovasc Pathol* **14**(2): 49-60.

Feinleib, M. (1976). "Discussion of: "Epidemiology of cardiovascular diseases: current perspectives"." *Am J Epidemiol* **104**(4): 457-62.

Fernandez-Catalan, C., W. Bode, et al. (1998). "Crystal structure of the complex formed by the membrane type 1-matrix metalloproteinase with the tissue inhibitor of metalloproteinases-2, the soluble progelatinase A receptor." *Embo J* **17**(17): 5238-48.

Ferrara, N., J. Winer, et al. (1993). "Expression of vascular endothelial growth factor does not promote transformation but confers a growth advantage in vivo to Chinese hamster ovary cells." *J Clin Invest* **91**(1): 160-70.

Ferrari, R., A. Cargnoni, et al. (1996). "Metabolic adaptation during a sequence of no-flow and low-flow ischemia. A possible trigger for hibernation." *Circulation* **94**(10): 2587-96.

Fini, M. (1998). Matrix Metalloproteinases. San Diego, Academic Press.

Fink, G., B. E. Sumner, et al. (1998). "Sex steroid control of mood, mental state and memory." *Clin Exp Pharmacol Physiol* **25**(10): 764-75.

Fliss, H. and D. Gattinger (1996). "Apoptosis in ischemic and reperfused rat myocardium." *Circ Res* **79**(5): 949-56.

Frangogiannis, N. G., C. W. Smith, et al. (2002). "The inflammatory response in myocardial infarction." *Cardiovasc Res* **53**(1): 31-47.

Frangogiannis, N. G., L. H. Michael, et al. (2000). "Myofibroblasts in reperfused myocardial infarcts express the embryonic form of smooth muscle myosin heavy chain (SMemb)." *Cardiovasc Res* **48**(1): 89-100.

Frasor, J., D. H. Barnett, et al. (2003). "Response-specific and ligand dose-dependent modulation of estrogen receptor (ER) alpha activity by ERbeta in the uterus." *Endocrinology* **144**(7): 3159-66.

Frasor, J., D. H. Barnett, et al. (2003). "Response-specific and ligand dose-dependent modulation of estrogen receptor (ER) alpha activity by ERbeta in the uterus." *Endocrinology* **144**(7): 3159-66.

Furie, M. B., M. C. Tancinco, et al. (1991). "Monoclonal antibodies to leukocyte integrins CD11a/CD18 and CD11b/CD18 or intercellular adhesion molecule-1 inhibit chemoattractant-stimulated neutrophil transendothelial migration in vitro." *Blood* **78**(8): 2089-97.

Furie, M. B., M. J. Burns, et al. (1992). "E-selectin (endothelial-leukocyte adhesion molecule-1) is not required for the migration of neutrophils across IL-1-stimulated endothelium in vitro." *J Immunol* **148**(8): 2395-404.

Gabel, S. A., V. R. Walker, et al. (2005). "Estrogen receptor beta mediates gender differences in ischemia/reperfusion injury." *J Mol Cell Cardiol* **38**(2): 289-97.

Gao, X. M., Q. Xu, et al. (2005). "Mouse model of post-infarct ventricular rupture: time course, strain- and gender-dependency, tensile strength, and histopathology." *Cardiovasc Res* **65**(2): 469-77.

Gerhard, M., B. W. Walsh, et al. (1998). "Estradiol therapy combined with progesterone and endothelium-dependent vasodilation in postmenopausal women." *Circulation* **98**(12): 1158-63.

Gilligan, D. M., D. M. Badar, et al. (1994). "Acute vascular effects of estrogen in postmenopausal women." *Circulation* **90**(2): 786-91.

Glasier, A. F., D. S. Irvine, et al. (1989). "A comparison of the effects on follicular development between clomiphene citrate, its two separate isomers and spontaneous cycles." *Hum Reprod* **4**(3): 252-6.

Goldberg, G. I., B. L. Marmer, et al. (1989). "Human 72-kilodalton type IV collagenase forms a complex with a tissue inhibitor of metalloproteases designated TIMP-2." *Proc Natl Acad Sci U S A* **86**(21): 8207-11.

Gomis-Ruth, F. X., K. Maskos, et al. (1997). "Mechanism of inhibition of the human matrix metalloproteinase stromelysin-1 by TIMP-1." *Nature* **389**(6646): 77-81.

Gorodeski, G. I., T. Yang, et al. (1998). "Modulation of coronary vascular resistance in female rabbits by estrogen and progesterone." *J Soc Gynecol Investig* **5**(4): 197-202.

Grady, D., D. Herrington, et al. (2002). "Cardiovascular disease outcomes during 6.8 years of hormone therapy: Heart and Estrogen/progestin Replacement Study follow-up (HERS II)." *Jama* **288**(1): 49-57.

Grimes, D. A. and R. A. Lobo (2002). "Perspectives on the Women's Health Initiative trial of hormone replacement therapy." *Obstet Gynecol* **100**(6): 1344-53.

Grodstein, F., J. E. Manson, et al. (2006). "Hormone therapy and coronary heart disease: the role of time since menopause and age at hormone initiation." *J Womens Health (Larchmt)* **15**(1): 35-44.

Grohe, C., S. Kahlert, et al. (1997). "Cardiac myocytes and fibroblasts contain functional estrogen receptors." *FEBS Lett* **416**(1): 107-12.

Grohe, C., S. Kahlert, et al. (1998). "Expression of oestrogen receptor alpha and beta in rat heart: role of local oestrogen synthesis." *J Endocrinol* **156**(2): R1-7.

Guerra, S., A. Leri, et al. (1999). "Myocyte death in the failing human heart is gender dependent." *Circ Res* **85**(9): 856-66.

Gustafsson, J. A. (1999). "Estrogen receptor beta--a new dimension in estrogen mechanism of action." *J Endocrinol* **163**(3): 379-83.

Hale, S. L., Y. Birnbaum, et al. (1996). "beta-Estradiol, but not alpha-estradiol, reduced myocardial necrosis in rabbits after ischemia and reperfusion." *Am Heart J* **132**(2 Pt 1): 258-62.

Hall, J. M. and D. P. McDonnell (1999). "The estrogen receptor beta-isoform (ERbeta) of the human estrogen receptor modulates ERalpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens." *Endocrinology* **140**(12): 5566-78.

Hall, J. M., J. F. Couse, et al. (2001). "The multifaceted mechanisms of estradiol and estrogen receptor signaling." *J Biol Chem* **276**(40): 36869-72.

Hanke, H., S. Hanke, et al. (1996). "Inhibition of the protective effect of estrogen by progesterone in experimental atherosclerosis." *Atherosclerosis* **121**(1): 129-38.

Hansen, P. R. (1995). "Role of neutrophils in myocardial ischemia and reperfusion." *Circulation* **91**(6): 1872-85.

Harman, S. M., E. A. Brinton, et al. (2005). "KEEPS: The Kronos Early Estrogen Prevention Study." *Climacteric* **8**(1): 3-12.

Hayashi, T., K. Yamada, et al. (1995). "Estrogen increases endothelial nitric oxide by a receptor-mediated system." *Biochem Biophys Res Commun* **214**(3): 847-55.

Hayashidani, S., H. Tsutsui, et al. (2003). "Targeted deletion of MMP-2 attenuates early LV rupture and late remodeling after experimental myocardial infarction." *Am J Physiol Heart Circ Physiol* **285**(3): H1229-35.

Heartstats.org. (2005). "British Heart Foundation Statistics website." from www.heartstats.org.

Helvering, L. M., M. D. Adrian, et al. (2005). "Differential effects of estrogen and raloxifene on messenger RNA and matrix metalloproteinase 2 activity in the rat uterus." *Biol Reprod* **72**(4): 830-41.

Helvering, L. M., M. D. Adrian, et al. (2005). "Differential effects of estrogen and raloxifene on messenger RNA and matrix metalloproteinase 2 activity in the rat uterus." *Biol Reprod* **72**(4): 830-41.

Henderson, B. E., A. Paganini-Hill, et al. (1991). "Decreased mortality in users of estrogen replacement therapy." *Arch Intern Med* **151**(1): 75-8.

Herrington, D. M. and K. P. Klein (2003). "Randomized clinical trials of hormone replacement therapy for treatment or prevention of cardiovascular disease: a review of the findings." *Atherosclerosis* **166**(2): 203-12.

Herrington, D. M. and T. D. Howard (2003b). "ER-alpha variants and the cardiovascular effects of hormone replacement therapy." *Pharmacogenomics* **4**(3): 269-77.

Herrington, D. M., D. M. Reboussin, et al. (2000). "Effects of estrogen replacement on the progression of coronary-artery atherosclerosis." *N Engl J Med* **343**(8): 522-9.

Herzog, E. G., A. Kohmoto, T. Burkhoff, D. Hochmann, JS (1998). "Early activation of metalloproteinases after experimental myocardial infarction occurs in the infarct and non-infarct zones." *Cardiol Pathol* **7**: 307-312.

Heymans, S., A. Luttun, et al. (1999). "Inhibition of plasminogen activators or matrix metalloproteinases prevents cardiac rupture but impairs therapeutic angiogenesis and causes cardiac failure." *Nat Med* **5**(10): 1135-42.

Heymans, S., A. Luttun, et al. (1999). "Inhibition of plasminogen activators or matrix metalloproteinases prevents cardiac rupture but impairs therapeutic angiogenesis and causes cardiac failure." *Nat Med* **5**(10): 1135-42.

Heyndrickx, G. R. (2006). "Early reperfusion phenomena." *Seimn Cardiothorac Vasc Anesth* **10**: 236.

Higashi, Y., M. Sanada, et al. (2001). "Effect of estrogen replacement therapy on endothelial function in peripheral resistance arteries in normotensive and hypertensive postmenopausal women." *Hypertension* **37**(2 Part 2): 651-7.

Hishikawa, K., T. Nakaki, et al. (1995). "Up-regulation of nitric oxide synthase by estradiol in human aortic endothelial cells." *FEBS Lett* **360**(3): 291-3.

Hodis, H. N., W. J. Mack, et al. (2001). "Estrogen in the prevention of atherosclerosis. A randomized, double-blind, placebo-controlled trial." *Ann Intern Med* **135**(11): 939-53.

Hornberger, L. K., S. Singhroy, et al. (2000). "Synthesis of extracellular matrix and adhesion through beta(1) integrins are critical for fetal ventricular myocyte proliferation." *Circ Res* **87**(6): 508-15.

Hudson, K. F. (1994). "A phenomenon of paradox: myocardial reperfusion injury." *Heart Lung* **23**(5): 384-93; quiz 394-6.

Hulley, S., D. Grady, et al. (1998). "Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. Heart and Estrogen/progestin Replacement Study (HERS) Research Group." *Jama* **280**(7): 605-13.

Hutchins, K. D., J. Skurnick, et al. (2002). "Cardiac rupture in acute myocardial infarction: a reassessment." *Am J Forensic Med Pathol* **23**(1): 78-82.

Ichihara, S., T. Senbonmatsu, et al. (2002). "Targeted deletion of angiotensin II type 2 receptor caused cardiac rupture after acute myocardial infarction." *Circulation* **106**(17): 2244-9.

Ingegno, M. D., S. R. Money, et al. (1988). "Progesterone receptors in the human heart and great vessels." *Lab Invest* **59**(3): 353-6.

Iruela-Arispe, M. L., P. Porter, et al. (1996). "Thrombospondin-1, an inhibitor of angiogenesis, is regulated by progesterone in the human endometrium." *J Clin Invest* **97**(2): 403-12.

Iruela-Arispe, M. L., P. Porter, et al. (1996). "Thrombospondin-1, an inhibitor of angiogenesis, is regulated by progesterone in the human endometrium." *J Clin Invest* **97**(2): 403-12.

Ito, I., T. Hayashi, et al. (1995). "Physiological concentration of estradiol inhibits polymorphonuclear leukocyte chemotaxis via a receptor mediated system." *Life Sci* **56**(25): 2247-53.

Ivey, C. L., F. M. Williams, et al. (1995). "Neutrophil chemoattractants generated in two phases during reperfusion of ischemic myocardium in the rabbit. Evidence for a role for C5a and interleukin-8." *J Clin Invest* **95**(6): 2720-8.

Jankowski, M., G. Rachelska, et al. (2001). "Estrogen receptors activate atrial natriuretic peptide in the rat heart." *Proc Natl Acad Sci U S A* **98**(20): 11765-70.

Jennings, R. B. and K. A. Reimer (1983). "Factors involved in salvaging ischemic myocardium: effect of reperfusion of arterial blood." *Circulation* **68**(2 Pt 2): I25-36.

Jennings, R. B. and K. A. Reimer (1991). "The cell biology of acute myocardial ischemia." *Annu Rev Med* **42**: 225-46.

Jeroudi, M. O., C. J. Hartley, et al. (1994). "Myocardial reperfusion injury: role of oxygen radicals and potential therapy with antioxidants." *Am J Cardiol* **73**(6): 2B-7B.

Jordan, J. E., Z. Q. Zhao, et al. (1999). "The role of neutrophils in myocardial ischemia-reperfusion injury." *Cardiovasc Res* **43**(4): 860-78.

Ju, H. and I. M. Dixon (1996). "Extracellular matrix and cardiovascular diseases." *Can J Cardiol* **12**(12): 1259-67.

Jugdutt, B. I. (1997). "Effect of reperfusion on ventricular mass, topography, and function during healing of anterior infarction." *Am J Physiol* **272**(3 Pt 2): H1205-11.

Jugdutt, B. I. (2003). "Ventricular remodeling after infarction and the extracellular collagen matrix: when is enough enough?" *Circulation* **108**(11): 1395-403.

Kalantaridou, S. N., K. K. Naka, et al. (2006). "Premature ovarian failure, endothelial dysfunction and estrogen-progestogen replacement." *Trends Endocrinol Metab* **17**(3): 101-9.

Kaminski, K. A., T. A. Bonda, et al. (2002). "Oxidative stress and neutrophil activation--the two keystones of ischemia/reperfusion injury." *Int J Cardiol* **86**(1): 41-59.

Kanekar, S., T. Hirozanne, et al. (1998). "Cardiacfibroblasts: form and function." *Cardiovasc Pathol* **7**(3): 127-133.

Karas, R. H., H. Schulten, et al. (2001). "Effects of estrogen on the vascular injury response in estrogen receptor alpha, beta (double) knockout mice." *Circ Res* **89**(6): 534-9.

Karas, R. H., J. B. Hodgin, et al. (1999). "Estrogen inhibits the vascular injury response in estrogen receptor beta-deficient female mice." *Proc Natl Acad Sci U S A* **96**(26): 15133-6.

Karas, R. H., M. van Eickels, et al. (2001). "A complex role for the progesterone receptor in the response to vascular injury." *J Clin Invest* **108**(4): 611-8.

Kawakami, R., Y. Saito, et al. (2004). "Overexpression of brain natriuretic peptide facilitates neutrophil infiltration and cardiac matrix metalloproteinase-9 expression after acute myocardial infarction." *Circulation* **110**(21): 3306-12.

Khreiss, T., L. Jozsef, et al. (2004). "Conformational rearrangement in C-reactive protein is required for proinflammatory actions on human endothelial cells." *Circulation* **109**(16): 2016-22.

Kim, H. P., J. Y. Lee, et al. (1999). "Nongenomic stimulation of nitric oxide release by estrogen is mediated by estrogen receptor alpha localized in caveolae." *Biochem Biophys Res Commun* **263**(1): 257-62.

Kim, J. K. and E. R. Levin (2006). "Estrogen signaling in the cardiovascular system." *Nucl Recept Signal* **4**: e013.

Kim, Y. D., B. Chen, et al. (1996). "17 beta-Estradiol prevents dysfunction of canine coronary endothelium and myocardium and reperfusion arrhythmias after brief ischemia/reperfusion." *Circulation* **94**(11): 2901-8.

Klebanoff, S. J., C. Mehlin, et al. (1997). "Activation of the HIV type 1 long terminal repeat and viral replication by dimethylsulfoxide and related solvents." *AIDS Res Hum Retroviruses* **13**(14): 1221-7.

Kloner, R. A., M. C. Fishbein, et al. (1978). "Mummification of the infarcted myocardium by high dose corticosteroids." *Circulation* **1**: 56-63.

Knauper, V., A. Osthues, et al. (1993). "Fragmentation of human polymorphonuclear-leucocyte collagenase." *Biochem J* **291 (Pt 3)**: 847-54.

Knauthe, R., P. Diel, et al. (1996). "Sexual dimorphism of steroid hormone receptor messenger ribonucleic acid expression and hormonal regulation in rat vascular tissue." *Endocrinology* **137**(8): 3220-7.

Knoepfler, P. S., C. M. Bloor, et al. (1995). "Urokinase plasminogen activator activity is increased in the myocardium during coronary artery occlusion." *J Mol Cell Cardiol* **27**(6): 1317-24.

Koh, K. K. and I. Sakuma (2004). "Should progestins be blamed for the failure of hormone replacement therapy to reduce cardiovascular events in randomized controlled trials?" *Arterioscler Thromb Vasc Biol* **24**(7): 1171-9.

Kolkenbrock, H., D. Orgel, et al. (1991). "The complex between a tissue inhibitor of metalloproteinases (TIMP-2) and 72-kDa progelatinase is a metalloproteinase inhibitor." *Eur J Biochem* **198**(3): 775-81.

Kolodgie, F. D., A. Farb, et al. (1997). "Myocardial protection of contractile function after global ischemia by physiologic estrogen replacement in the ovariectomized rat." *J Mol Cell Cardiol* **29**(9): 2403-14.

Korte, T., M. Fuchs, et al. (2005). "Female mice lacking estrogen receptor beta display prolonged ventricular repolarization and reduced ventricular automaticity after myocardial infarction." *Circulation* **111**(18): 2282-90.

Krege, J. H., J. B. Hodgin, et al. (1998). "Generation and reproductive phenotypes of mice lacking estrogen receptor beta." *Proc Natl Acad Sci U S A* **95**(26): 15677-82.

Kronos, L. R. I. (2006). " Kronos Longevity Research Institute." from www.kronosinstitute.org.

Kuiper, G. G., B. Carlsson, et al. (1997). "Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta." *Endocrinology* **138**(3): 863-70.

Kuiper, G. G., E. Enmark, et al. (1996). "Cloning of a novel receptor expressed in rat prostate and ovary." *Proc Natl Acad Sci U S A* **93**(12): 5925-30.

Kukielka, G. L., C. W. Smith, et al. (1995a). "Interleukin-8 gene induction in the myocardium after ischemia and reperfusion in vivo." *J Clin Invest* **95**(1): 89-103.

Kukielka, G. L., C. W. Smith, et al. (1995b). "Induction of interleukin-6 synthesis in the myocardium. Potential role in postreperfusion inflammatory injury." *Circulation* **92**(7): 1866-75.

Kushawaha, R. (1992). "Female sex steroid hormones and lipoprotein metabolism." *Curr Opinion Lipid* **3**: 167-172.

Kusuoka, H., J. K. Porterfield, et al. (1987). "Pathophysiology and pathogenesis of stunned myocardium. Depressed Ca2+ activation of contraction as a consequence of

reperfusion-induced cellular calcium overload in ferret hearts." *J Clin Invest* **79**(3): 950-61.

Lakoski, S. G. and D. M. Herrington (2005). "Effects of hormone therapy on C-reactive protein and IL-6 in postmenopausal women: a review article." *Climacteric* **8**(4): 317-26.

Langer, R. D. (2002). "Hormone replacement and the prevention of cardiovascular disease." *Am J Cardiol* **89**(12A): 36E-46E; discussion 46E.

Lawrence, M. B. and T. A. Springer (1991). "Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins." *Cell* **65**(5): 859-73.

Lazdunski, M., C. Frelin, et al. (1985). "The sodium/hydrogen exchange system in cardiac cells: its biochemical and pharmacological properties and its role in regulating internal concentrations of sodium and internal pH." *J Mol Cell Cardiol* **17**(11): 1029-42.

Lee, H. W. and M. Eghbali-Webb (1998). "Estrogen enhances proliferative capacity of cardiac fibroblasts by estrogen receptor- and mitogen-activated protein kinase-dependent pathways." *J Mol Cell Cardiol* **30**(7): 1359-68.

Lee, H. W. and M. Eghbali-Webb (1998). "Estrogen enhances proliferative capacity of cardiac fibroblasts by estrogen receptor- and mitogen-activated protein kinase-dependent pathways." *J Mol Cell Cardiol* **30**(7): 1359-68.

Lee, S. H., P. L. Wolf, et al. (2000). "Early expression of angiogenesis factors in acute myocardial ischemia and infarction." *N Engl J Med* **342**(9): 626-33.

Lefer, A. M. (1987). "Physiologic and pathophysiologic role of cyclo-oxygenase metabolites of arachidonic acid in circulatory disease states." *Cardiovasc Clin* **18**(1): 85-99.

Leiberman, J. R., D. Fraser, et al. (1995). "Breech presentation and cesarean section in term nulliparous women." *Eur J Obstet Gynecol Reprod Biol* **61**(2): 111-5.

Leibovich, S. J. and R. Ross (1975). "The role of the macrophage in wound repair. A study with hydrocortisone and antimacrophage serum." *Am J Pathol* **78**(1): 71-100.

Lerner, D. J. and W. B. Kannel (1986). "Patterns of coronary heart disease morbidity and mortality in the sexes: a 26-year follow-up of the Framingham population." *Am Heart J* **111**(2): 383-90.

Lesnefsky, E. J., S. Moghaddas, et al. (2001). "Mitochondrial dysfunction in cardiac disease: ischemia--reperfusion, aging, and heart failure." *J Mol Cell Cardiol* **33**(6): 1065-89.

Levine, R. L., S. J. Chen, et al. (1996). "Medroxyprogesterone attenuates estrogen-mediated inhibition of neointima formation after balloon injury of the rat carotid artery." *Circulation* **94**(9): 2221-7.

Lewis, A. J., H. B. Burchell, et al. (1969). "Clinical and pathologic features of postinfarction cardiac rupture." *Am J Cardiol* **23**(1): 43-53.

Li, J., L. F. Brown, et al. (1996). "VEGF, flk-1, and flt-1 expression in a rat myocardial infarction model of angiogenesis." *Am J Physiol* **270**(5 Pt 2): H1803-11.

Liao, F., H. K. Huynh, et al. (1995). "Migration of monocytes across endothelium and passage through extracellular matrix involve separate molecular domains of PECAM-1." *J Exp Med* **182**(5): 1337-43.

Libby, P., P. R. Maroko, et al. (1973). "Reduction of experimental myocardial infarct size by corticosteroid administration." *J Clin Invest* **3**: 599-607.

Lichtinghagen, R., D. Michels, et al. (2001). "Matrix metalloproteinase (MMP)-2, MMP-7, and tissue inhibitor of metalloproteinase-1 are closely related to the fibroproliferative process in the liver during chronic hepatitis C." *J Hepatol* **34**(2): 239-47.

Lima, S. M., J. M. Aldrighi, et al. (2005). "Acute administration of 17beta-estradiol improves endothelium-dependent vasodilation in postmenopausal women." *Maturitas* **50**(4): 266-74.

Lindsey, M. L., J. Gannon, et al. (2002). "Selective matrix metalloproteinase inhibition reduces left ventricular remodeling but does not inhibit angiogenesis after myocardial infarction." *Circulation* **105**(6): 753-8.

Lindsey, M., K. Wedin, et al. (2001). "Matrix-dependent mechanism of neutrophil-mediated release and activation of matrix metalloproteinase 9 in myocardial ischemia/reperfusion." *Circulation* **103**(17): 2181-7.

Losel, R. and M. Wehling (2003). "Nongenomic actions of steroid hormones." *Nat Rev Mol Cell Biol* **4**(1): 46-56.

Losordo, D. W., M. Kearney, et al. (1994). "Variable expression of the estrogen receptor in normal and atherosclerotic coronary arteries of premenopausal women." *Circulation* **89**(4): 1501-10.

Lovelock, J. D., A. H. Baker, et al. (2005). "Heterogeneous effects of tissue inhibitors of matrix metalloproteinases on cardiac fibroblasts." *Am J Physiol Heart Circ Physiol* **288**(2): H461-8.

Lu, Q., D. C. Pallas, et al. (2004). "Striatin assembles a membrane signaling complex necessary for rapid, nongenomic activation of endothelial NO synthase by estrogen receptor alpha." *Proc Natl Acad Sci U S A* **101**(49): 17126-31.

Lu, T., Y. Achari, et al. (2006). "Estrogen receptor alpha regulates matrix metalloproteinase-13 promoter activity primarily through the AP-1 transcriptional regulatory site." *Biochim Biophys Acta* **1762**(8): 719-31.

Lucchesi, B. R. (1990). "Modulation of leukocyte-mediated myocardial reperfusion injury." *Annu Rev Physiol* **52**: 561-76.

Lukashev, M. E. and Z. Werb (1998). "ECM signalling: orchestrating cell behaviour and misbehaviour." *Trends Cell Biol* **8**(11): 437-41.

Mahmoodzadeh, S., S. Eder, et al. (2006). "Estrogen receptor alpha up-regulation and redistribution in human heart failure." *Faseb J* **20**(7): 926-34.

Mann, D. L. and F. G. Spinale (1998). "Activation of matrix metalloproteinases in the failing human heart: breaking the tie that binds." *Circulation* **98**(17): 1699-702.

Manson, J. E., J. Hsia, et al. (2003). "Estrogen plus progestin and the risk of coronary heart disease." *N Engl J Med* **349**(6): 523-34.

Manson, J. E., S. S. Bassuk, et al. (2006). "Postmenopausal hormone therapy: new questions and the case for new clinical trials." *Menopause* **13**(1): 139-47.

Marbaix, E., J. Donnez, et al. (1992). "Progesterone regulates the activity of collagenase and related gelatinases A and B in human endometrial explants." *Proc Natl Acad Sci U S A* **89**(24): 11789-93.

Mathey, D. G., F. H. Sheehan, et al. (1985). "Time from onset of symptoms to thrombolytic therapy: a major determinant of myocardial salvage in patients with acute transmural infarction." *J Am Coll Cardiol* **6**(3): 518-25.

Mathrubutham, M. and K. Fotherby (1981). "Medroxyprogesterone acetate in human serum." *J Steroid Biochem* **14**(8): 783-6.

Matrisian, L. M. (1992). "The matrix-degrading metalloproteinases." *Bioessays* **14**(7): 455-63.

McCord, J. M. (1985). "Oxygen-derived free radicals in postischemic tissue injury." *N Engl J Med* **312**(3): 159-63.

McEwan, B. S. (2002). "Estrogen actions throughout the brain." *Recent Prog. Horm. Res* **57**: 357-384.

McHugh, N. A., G. F. Merrill, et al. (1998). "Estrogen diminishes postischemic hydroxyl radical production." *Am J Physiol* **274**(6 Pt 2): H1950-4.

McNeill, A. M., C. Zhang, et al. (2002). "Estrogen increases endothelial nitric oxide synthase via estrogen receptors in rat cerebral blood vessels: effect preserved after concurrent treatment with medroxyprogesterone acetate or progesterone." *Stroke* **33**(6): 1685-91.

Meade, T. W. and A. Berra (1992). "Hormone replacement therapy and cardiovascular disease." *Br Med Bull* **48**(2): 276-308.

Mendelsohn, M. E. (2002). "Protective effects of estrogen on the cardiovascular system." *Am J Cardiol* **89**(12A): 12E-17E; discussion 17E-18E.

Mendelsohn, M. E. and R. H. Karas (1994). "Estrogen and the blood vessel wall." *Curr Opin Cardiol* **9**(5): 619-26.

Mendelsohn, M. E. and R. H. Karas (1999). "The protective effects of estrogen on the cardiovascular system." *N Engl J Med* **340**(23): 1801-11.

Mendelsohn, M. E. and R. H. Karas (2005). "Molecular and cellular basis of cardiovascular gender differences." *Science* **308**(5728): 1583-7.

Mensah-Brown, E. P., D. E. Rizk, et al. (2004). "Effects of ovariectomy and hormone replacement on submucosal collagen and blood vessels of the anal canal of rats." *Colorectal Dis* **6**(6): 481-7.

Mercuro, G., G. Longu, et al. (1999a). "Impaired forearm blood flow and vasodilator reserve in healthy postmenopausal women." *Am Heart J* **137**(4 Pt 1): 692-7.

Mercuro, G., L. Pitzalis, et al. (1999). "Effects of acute administration of natural progesterone on peripheral vascular responsiveness in healthy postmenopausal women." *Am J Cardiol* **84**(2): 214-8.

Mignatti, P.

Minshall, R. D., F. Z. Stanczyk, et al. (1998). "Ovarian steroid protection against coronary artery hyperreactivity in rhesus monkeys." *J Clin Endocrinol Metab* **83**(2): 649-59.

Miyamori, H., T. Takino, et al. (2000). "Human membrane type-2 matrix metalloproteinase is defective in cell-associated activation of progelatinase A." *Biochem Biophys Res Commun* **267**(3): 796-800.

Moncada, S. and A. Higgs (1993). "The L-arginine-nitric oxide pathway." *N Engl J Med* **329**(27): 2002-12.

Mosca, L., E. Barrett-Connor, et al. (2001). "Design and methods of the Raloxifene Use for The Heart (RUTH) study." *Am J Cardiol* **88**(4): 392-5.

Mosca, L., L. J. Appel, et al. (2004). "Summary of the American Heart Association's evidence-based guidelines for cardiovascular disease prevention in women." *Arterioscler Thromb Vasc Biol* **24**(3): 394-6.

Moscatelli, D. and D. B. Rifkin (1988). "Membrane and matrix localization of proteinases: a common theme in tumor cell invasion and angiogenesis." *Biochim Biophys Acta* **948**(1): 67-85.

Mosselman, S., J. Polman, et al. (1996). "ER beta: identification and characterization of a novel human estrogen receptor." *FEBS Lett* **392**(1): 49-53.

Mott, J. D. and Z. Werb (2004). "Regulation of matrix biology by matrix metalloproteinases." *Curr Opin Cell Biol* **16**(5): 558-64.

Muddana, S. S. and B. R. Peterson (2003). "Fluorescent cellular sensors of steroid receptor ligands." *Chembiochem* **4**(9): 848-55.

Mullane, K. M. and A. Pinto (1987). "Endothelium, arachidonic acid, and coronary vascular tone." *Fed Proc* **46**(1): 54-62.

Muller, W. A., S. A. Weigl, et al. (1993). "PECAM-1 is required for transendothelial migration of leukocytes." *J Exp Med* **178**(2): 449-60.

Munshi, H. G., Y. I. Wu, et al. (2004). "Differential regulation of membrane type 1-matrix metalloproteinase activity by ERK 1/2- and p38 MAPK-modulated tissue inhibitor of metalloproteinases 2 expression controls transforming growth factor-beta1-induced pericellular collagenolysis." *J Biol Chem* **279**(37): 39042-50.

Murphy, P. M. (1994). "The molecular biology of leukocyte chemoattractant receptors." *Annu Rev Immunol* **12**: 593-633.

Nagaoka, I. and S. Hirota (2000). "Increased expression of matrix metalloproteinase-9 in neutrophils in glycogen-induced peritoneal inflammation of guinea pigs." *Inflamm Res* **49**(2): 55-62.

Nagase, H. and J. F. Woessner, Jr. (1999). "Matrix metalloproteinases." *J Biol Chem* **274**(31): 21491-4.

Nahrendorf, M., K. Hu, et al. (2006). "Factor XIII deficiency causes cardiac rupture, impairs wound healing, and aggravates cardiac remodeling in mice with myocardial infarction." *Circulation* **113**(9): 1196-202.

Nakamura, Y., T. Suzuki, et al. (2004). "Estrogen receptors in atherosclerotic human aorta: inhibition of human vascular smooth muscle cell proliferation by estrogens." *Mol Cell Endocrinol* **219**(1-2): 17-26.

Nakano, T., T. Konishi, et al. (1985). "Potential prevention of myocardial rupture resulting from acute myocardial infarction." *Clin Cardiol* **8**(4): 199-204.

Ness, K. F. (2005). Distribution and function of the oestroegn receptor beta in the cardiovascular system of the mouse. Centre for Cardiovascular Science, University of Edinburgh. **PhD**.

Nilsson, S., S. Makela, et al. (2001). "Mechanisms of estrogen action." *Physiol Rev* **81**(4): 1535-65.

Nuedling, S., R. H. Karas, et al. (2001). "Activation of estrogen receptor beta is a prerequisite for estrogen-dependent upregulation of nitric oxide synthases in neonatal rat cardiac myocytes." *FEBS Lett* **502**(3): 103-8.

Ogawa, E., Y. Saito, et al. (2000). "Outside-in signalling of fibronectin stimulates cardiomyocyte hypertrophy in cultured neonatal rat ventricular myocytes." *J Mol Cell Cardiol* **32**(5): 765-76.

Ohuchi, E., K. Imai, et al. (1997). "Membrane type 1 matrix metalloproteinase digests interstitial collagens and other extracellular matrix macromolecules." *J Biol Chem* **272**(4): 2446-51.

Oishi, A., M. Ohmichi, et al. (2004). "Medroxyprogesterone acetate attenuates estrogen-induced nitric oxide production in human umbilical vein endothelial cells." *Biochem Biophys Res Commun* **324**(1): 193-8.

O'Rourke, M. F. (1973). "Subacute heart rupture following myocardial infarction. Clinical features of a correctable condition." *Lancet* **2**(7821): 124-6.

O'Shaughnessy, A., O. Muneyyirci-Delale, et al. (2001). "Circulating divalent cations in asymptomatic ovarian hyperstimulation and in vitro fertilization patients." *Gynecol Obstet Invest* **52**(4): 237-42.

Ottoson, U. B., K. Carlstrom, et al. (1984). "Serum levels of progesterone and some of its metabolites including deoxycorticosterone after oral and parenteral administration." *Br J Obstet Gynaecol* **91**(11): 1111-9.

Pap, T., Y. Shigeyama, et al. (2000). "Differential expression pattern of membrane-type matrix metalloproteinases in rheumatoid arthritis." *Arthritis Rheum* **43**(6): 1226-32.

Pappas, T. C., B. Gametchu, et al. (1995). "Membrane estrogen receptors identified by multiple antibody labeling and impeded-ligand binding." *Faseb J* **9**(5): 404-10.

Park, J. L. and B. R. Lucchesi (1999). "Mechanisms of myocardial reperfusion injury." *Ann Thorac Surg* **68**(5): 1905-12.

Parker, M. G. (1995). "Structure and function of estrogen receptors." *Vitam Horm* **51**: 267-87.

Patten, R. D. and R. H. Karas (2006). "Estrogen replacement and cardiomyocyte protection." *Trends Cardiovasc Med* **16**(3): 69-75.

Paul, B. B., R. R. Strauss, et al. (1970). "Function of H(2)O(2), Myeloperoxidase, and Hexose Monophosphate Shunt Enzymes in Phagocytizing Cells from Different Species." *Infect Immun* **1**(4): 338-344.

Pei, D. and S. J. Weiss (1995). "Furin-dependent intracellular activation of the human stromelysin-3 zymogen." *Nature* **375**(6528): 244-7.

Pelzer, T., V. Jazbutyte, et al. (2005). "The estrogen receptor-alpha agonist 16alpha-LE2 inhibits cardiac hypertrophy and improves hemodynamic function in estrogen-deficient spontaneously hypertensive rats." *Cardiovasc Res* **67**(4): 604-12.

Pepys, M. B., G. M. Hirschfield, et al. (2006). "Targeting C-reactive protein for the treatment of cardiovascular disease." *Nature* **440**(7088): 1217-21.

Persky, A. M., P. S. Green, et al. (2000). "Protective effect of estrogens against oxidative damage to heart and skeletal muscle in vivo and in vitro." *Proc Soc Exp Biol Med* **223**(1): 59-66.

Peterson, J. T., H. Li, et al. (2000). "Evolution of matrix metalloprotease and tissue inhibitor expression during heart failure progression in the infarcted rat." *Cardiovasc Res* **46**(2): 307-15.

Petrov, V. V., R. H. Fagard, et al. (2002). "Stimulation of collagen production by transforming growth factor-beta1 during differentiation of cardiac fibroblasts to myofibroblasts." *Hypertension* **39**(2): 258-63.

Pfeffer, M. A. and E. Braunwald (1990). "Ventricular remodeling after myocardial infarction. Experimental observations and clinical implications." *Circulation* **81**(4): 1161-72.

Pietras, R. J. and C. M. Szego (1977). "Specific binding sites for oestrogen at the outer surfaces of isolated endometrial cells." *Nature* **265**(5589): 69-72.

Pietras, R. J. and C. M. Szego (1980). "Partial purification and characterization of oestrogen receptors in subfractions of hepatocyte plasma membranes." *Biochem J* **191**(3): 743-60.

Pike, A. C., A. M. Brzozowski, et al. (1999). "Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist." *Embo J* **18**(17): 4608-18.

Pike, A. C., A. M. Brzozowski, et al. (2001). "Structural insights into the mode of action of a pure antiestrogen." *Structure (Camb)* **9**(2): 145-53.

Post, M. S., M. Christella, et al. (2003). "Effect of oral and transdermal estrogen replacement therapy on hemostatic variables associated with venous thrombosis: a randomized, placebo-controlled study in postmenopausal women." *Arterioscler Thromb Vasc Biol* **23**(6): 1116-21.

Potier, M., S. J. Elliot, et al. (2001). "Expression and regulation of estrogen receptors in mesangial cells: influence on matrix metalloproteinase-9." *J Am Soc Nephrol* **12**(2): 241-51.

Premaratne, S., W. Limm, et al. (1993). "Quantitative assessment of free-radical generation during ischemia and reperfusion in the isolated rabbit heart." *Coron Artery Dis* **4**(12): 1093-6.

Proudler, A. J., A. I. Ahmed, et al. (1995). "Hormone replacement therapy and serum angiotensin-converting-enzyme activity in postmenopausal women." *Lancet* **346**(8967): 89-90.

Quyyumi, A. A. (2006). "Women and ischemic heart disease: pathophysiologic implications from the Women's Ischemia Syndrome Evaluation (WISE) Study and future research steps." *J Am Coll Cardiol* **47**(3 Suppl): S66-71.

Rapti, M., V. Knauper, et al. (2006). "Characterization of the AB loop region of TIMP-2. Involvement in pro-MMP-2 activation." *J Biol Chem* **281**(33): 23386-94.

Razandi, M., A. Pedram, et al. (1999). "Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: studies of ERalpha and ERbeta expressed in Chinese hamster ovary cells." *Mol Endocrinol* **13**(2): 307-19.

Reddy, S. G. and W. C. Roberts (1989). "Frequency of rupture of the left ventricular free wall or ventricular septum among necropsy cases of fatal acute myocardial infarction since introduction of coronary care units." *Am J Cardiol* **63**(13): 906-11.

Reimer, K. A., C. E. Murry, et al. (1989). "The role of neutrophils and free radicals in the ischemic-reperfused heart: why the confusion and controversy?" *J Mol Cell Cardiol* **21**(12): 1225-39.

Reynaud, C., C. Gleyzal, et al. (1999). "Comparative functional study of the lysyl oxidase promoter in fibroblasts, Ras-transformed fibroblasts, myofibroblasts and smooth muscle cells." *Cell Mol Biol (Noisy-le-grand)* **45**(8): 1237-47.

Riley, S. C., A. H. Gibson, et al. (2001). "Secretion of matrix metalloproteinases 2 and 9 and tissue inhibitor of metalloproteinases into follicular fluid during follicle development in equine ovaries." *Reproduction* **121**(4): 553-60.

Riley, S. C., R. Leask, et al. (1999). "Secretion of matrix metalloproteinase-2, matrix metalloproteinase-9 and tissue inhibitor of metalloproteinases into the intrauterine compartments during early pregnancy." *Mol Hum Reprod* **5**(4): 376-81.

Robinson, T. F., L. Cohen-Gould, et al. (1983). "Skeletal framework of mammalian heart muscle. Arrangement of inter- and pericellular connective tissue structures." *Lab Invest* **49**(4): 482-98.

Roesch, D. M. (2006). "Effects of selective estrogen receptor agonists on food intake and body weight gain in rats." *Physiol Behav* **87**(1): 39-44.

Rohde, L. E., A. Ducharme, et al. (1999). "Matrix metalloproteinase inhibition attenuates early left ventricular enlargement after experimental myocardial infarction in mice." *Circulation* **99**(23): 3063-70.

Romanic, A. M., C. L. Burns-Kurtis, et al. (2001). "Matrix metalloproteinase expression in cardiac myocytes following myocardial infarction in the rabbit." *Life Sci* **68**(7): 799-814.

Ross, R. and J. A. Glomset (1973). "Atherosclerosis and the arterial smooth muscle cell: Proliferation of smooth muscle is a key event in the genesis of the lesions of atherosclerosis." *Science* **180**(93): 1332-9.

Ross, R. S. and T. K. Borg (2001). "Integrins and the myocardium." *Circ Res* **88**(11): 1112-9.

Rossen, R. D., L. H. Michael, et al. (1994). "Cardiolipin-protein complexes and initiation of complement activation after coronary artery occlusion." *Circ Res* **75**(3): 546-55.

Rossouw, J. E., G. L. Anderson, et al. (2002). "Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial." *Jama* **288**(3): 321-33.

Rubanyi, G. M., A. D. Freay, et al. (1997). "Vascular estrogen receptors and endothelium-derived nitric oxide production in the mouse aorta. Gender difference and effect of estrogen receptor gene disruption." *J Clin Invest* **99**(10): 2429-37.

Ruehlmann, D. O. and G. E. Mann (1997). "Actions of oestrogen on vascular endothelial and smooth-muscle cells." *Biochem Soc Trans* **25**(1): 40-5.

Ruff, M., M. Gangloff, et al. (2000). "Estrogen receptor transcription and transactivation: Structure-function relationship in DNA- and ligand-binding domains of estrogen receptors." *Breast Cancer Res* **2**(5): 353-9.

Sackner-Bernstein, J. D. (2000). "The myocardial matrix and the development and progression of ventricular remodeling." *Curr Cardiol Rep* **2**(2): 112-9.

Saksela, O. and D. B. Rifkin (1988). "Cell-associated plasminogen activation: regulation and physiological functions." *Annu Rev Cell Biol* **4**: 93-126.

Salpeter, S. R., J. M. Walsh, et al. (2004). "Mortality associated with hormone replacement therapy in younger and older women: a meta-analysis." *J Gen Intern Med* **19**(7): 791-804.

Sato, H., T. Kinoshita, et al. (1996). "Activation of a recombinant membrane type 1-matrix metalloproteinase (MT1-MMP) by furin and its interaction with tissue inhibitor of metalloproteinases (TIMP)-2." *FEBS Lett* **393**(1): 101-4.

Saunders, P. T., S. M. Maguire, et al. (1997). "Expression of oestrogen receptor beta (ER beta) in multiple rat tissues visualised by immunohistochemistry." *J Endocrinol* **154**(3): R13-6.

Schrepfer, S., T. Deuse, et al. (2006). "The selective estrogen receptor-beta agonist biochanin A shows vasculoprotective effects without uterotrophic activity." *Menopause* **13**(3): 489-99.

Seals, D. R., K. L. Moreau, et al. (2006). "Modulatory influences on ageing of the vasculature in healthy humans." *Exp Gerontol* **41**(5): 501-7.

Seeger, H., D. Wallwiener, et al. (2005). "Different effects of estradiol and various antiestrogens on TNF-alpha-induced changes of biochemical markers for growth and invasion of human breast cancer cells." *Life Sci.*

Sekido, N., N. Mukaida, et al. (1993). "Prevention of lung reperfusion injury in rabbits by a monoclonal antibody against interleukin-8." *Nature* **365**(6447): 654-7.

Selye, H., E. Bajusz, et al. (1960). "Simple techniques for the surgical occlusion of coronary vessels in the rat." *Angiology* **11**: 398-407.

Sharif, I. (2002). "Oestrogen worsens outcome following myocardial infarction in mice. ." *Br J Pharmacol* **137**: 4P.

Sherry, L. (2000). Investigation of cardiac endothelin system post MI in the rat. Edinburgh, University of Edinburgh. **PhD**.

Shi, W., M. D. Brown, et al. (2003). "Genetic backgrounds but not sizes of atherosclerotic lesions determine medial destruction in the aortic root of apolipoprotein E-deficient mice." *Arterioscler Thromb Vasc Biol* **23**(10): 1901-6.

Shingu, M. and M. Nobunaga (1984). "Chemotactic activity generated in human serum from the fifth component of complement by hydrogen peroxide." *Am J Pathol* **2**: 201-206.

Simms, M. G. and K. R. Walley (1999). "Activated macrophages decrease rat cardiac myocyte contractility: importance of ICAM-1-dependent adhesion." *Am J Physiol* **277**(1 Pt 2): H253-60.

Simoncini, T., P. Mannella, et al. (2003). "In vitro effects of progesterone and progestins on vascular cells." *Steroids* **68**(10-13): 831-6.

Simoncini, T., P. Mannella, et al. (2004). "Differential signal transduction of progesterone and medroxyprogesterone acetate in human endothelial cells." *Endocrinology* **145**(12): 5745-56.

Simoncini, T., P. Mannella, et al. (2004b). "Genomic and non-genomic effects of estrogens on endothelial cells." *Steroids* **69**(8-9): 537-42.

Simpson, D. G., L. Terracio, et al. (1994). "Modulation of cardiac myocyte phenotype in vitro by the composition and orientation of the extracellular matrix." *J Cell Physiol* **161**(1): 89-105.

Small, G. R., P. W. Hadoke, et al. (2005). "Preventing local regeneration of glucocorticoids by 11beta-hydroxysteroid dehydrogenase type 1 enhances angiogenesis." *Proc Natl Acad Sci U S A* **102**(34): 12165-70.

Smith, J. B., C. Dangelmaier, et al. (1992). "Cyclic AMP does not inhibit collagen-induced platelet signal transduction." *Biochem J* **283 (Pt 3)**: 889-92.

Smith, P. J., O. Ornatsky, et al. (2000). "Effects of estrogen replacement on infarct size, cardiac remodeling, and the endothelin system after myocardial infarction in ovariectomized rats." *Circulation* **102**(24): 2983-9.

Smithson, G., J. F. Couse, et al. (1998). "The role of estrogen receptors and androgen receptors in sex steroid regulation of B lymphopoiesis." *J Immunol* **161**(1): 27-34.

Solomon, S. D. and M. A. Pfeffer (2002). "Renin-angiotensin system and cardiac rupture after myocardial infarction." *Circulation* **106**(17): 2167-9.

Somunkiran, A., B. Yazici, et al. (2006). "Effects of tibolone on blood flow resistance and intima-media thickness of the carotid arteries: effect of time since menopause." *Climacteric* **9**(1): 59-65.

Spinale, F. G. (2002). "Matrix metalloproteinases: regulation and dysregulation in the failing heart." *Circ Res* **90**(5): 520-30.

Spinale, F. G., F. A. Crawford, Jr., et al. (1991). "Ventricular failure and cellular remodeling with chronic supraventricular tachycardia." *J Thorac Cardiovasc Surg* **102**(6): 874-82.

Spinale, F. G., M. L. Coker, et al. (2000). "Myocardial matrix degradation and metalloproteinase activation in the failing heart: a potential therapeutic target." *Cardiovasc Res* **46**(2): 225-38.

Springer, T. A. (1994). "Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm." *Cell* **76**(2): 301-14.

Squadrito, F., D. Altavilla, et al. (1997). "17Beta-oestradiol reduces cardiac leukocyte accumulation in myocardial ischaemia reperfusion injury in rat." *Eur J Pharmacol* **335**(2-3): 185-92.

Squires, C. E., G. P. Escobar, et al. (2005). "Altered fibroblast function following myocardial infarction." *J Mol Cell Cardiol* **39**(4): 699-707.

Stampfer, M. J. and G. A. Colditz (1991). "Estrogen replacement therapy and coronary heart disease: a quantitative assessment of the epidemiologic evidence." *Prev Med* **20**(1): 47-63.

Stampfer, M. J., W. C. Willett, et al. (1985). "A prospective study of postmenopausal estrogen therapy and coronary heart disease." *N Engl J Med* **313**(17): 1044-9.

Stancovski, I. and D. Baltimore (1997). "NF-kappaB activation: the I kappaB kinase revealed?" *Cell* **91**(3): 299-302.

Steinberg, K. K., S. B. Thacker, et al. (1991). "A meta-analysis of the effect of estrogen replacement therapy on the risk of breast cancer." *Jama* **265**(15): 1985-90.

Stevenson, J. C. (2000). "Cardiovascular effects of oestrogens." *J Steroid Biochem Mol Biol* **74**(5): 387-93.

Stevenson, J. C. (2004). "The metabolic basis for the effects of HRT on coronary heart disease." *Endocrine* **24**(3): 239-44.

Stirone, C., S. P. Duckles, et al. (2005). "Estrogen increases mitochondrial efficiency and reduces oxidative stress in cerebral blood vessels." *Mol Pharmacol* **68**(4): 959-65.

Strehlow, K., S. Rotter, et al. (2003). "Modulation of antioxidant enzyme expression and function by estrogen." *Circ Res* **93**(2): 170-7.

Stumpf, W. E., M. Sar, et al. (1977). "The heart: a target organ for estradiol." *Science* **196**(4287): 319-21.

Sturdee, D. W. (1999). "Current hormone replacement therapy: what are the shortcomings? Advances in delivery." *Int J Clin Pract* **53**(6): 468-72.

Stygar, D., P. Westlund, et al. (2006). "Identification of wild type and variants of oestrogen receptors in polymorphonuclear and mononuclear leucocytes." *Clin Endocrinol (Oxf)* **64**(1): 74-81.

Sudhir, K., M. D. Elser, et al. (1997). "Estrogen supplementation decreases norepinephrine-induced vasoconstriction and total body norepinephrine spillover in perimenopausal women." *Hypertension* **30**(6): 1538-43.

Sugioka, K., Y. Shimosegawa, et al. (1987). "Estrogens as natural antioxidants of membrane phospholipid peroxidation." *FEBS Lett* **210**(1): 37-9.

Sullivan, T. R., Jr., R. H. Karas, et al. (1995). "Estrogen inhibits the response-to-injury in a mouse carotid artery model." *J Clin Invest* **96**(5): 2482-8.

Sun, M., F. Dawood, et al. (2004). "Excessive tumor necrosis factor activation after infarction contributes to susceptibility of myocardial rupture and left ventricular dysfunction." *Circulation* **110**(20): 3221-8.

Sun, P., L. Wei, et al. (2006). "The orphan nuclear receptors, estrogen receptor-related receptors: their role as new biomarkers in gynecological cancer." *Anticancer Res* **26**(2C): 1699-706.

Sun, Y. and K. T. Weber (2000b). "Infarct scar: a dynamic tissue." *Cardiovasc Res* **46**(2): 250-6.

Sun, Y., J. Q. Zhang, et al. (2000a). "Cardiac remodeling by fibrous tissue after infarction in rats." *J Lab Clin Med* **135**(4): 316-23.

Takano, H., H. Hasegawa, et al. (2003). "Implication of cardiac remodeling in heart failure: mechanisms and therapeutic strategies." *Intern Med* **42**(6): 465-9.

Tan, E., M. V. Gurjar, et al. (1999). "Estrogen receptor-alpha gene transfer into bovine aortic endothelial cells induces eNOS gene expression and inhibits cell migration." *Cardiovasc Res* **43**(3): 788-97.

Tao, Z. Y., M. A. Cavasin, et al. (2004). "Temporal changes in matrix metalloproteinase expression and inflammatory response associated with cardiac rupture after myocardial infarction in mice." *Life Sci* **74**(12): 1561-72.

Tauber, A. I. and B. M. Babior (1977). "Evidence for hydroxyl radical production by human neutrophils." *J Clin Invest* **60**(2): 374-9.

Taylor, A. H. and F. Al-Azzawi (2000). "Immunolocalisation of oestrogen receptor beta in human tissues." *J Mol Endocrinol* **24**(1): 145-55.

Taylor, A. H., A. E. Fox-Robichaud, et al. (2000). "Oestradiol decreases rat apolipoprotein AI transcription via promoter site B." *J Mol Endocrinol* **25**(2): 207-19.

The Women's Health Initiative Study Group (1998). "Design of the Women's Health Initiative clinical trial and observational study. ." *Control Clin Trials* **19**(1): 61-109.

The writing group for the PEPI trial (1995). "The effect of estrogen or estrogen/progestin on heart disease risk factors in postmenopausal women: The Postmenopausal Estrogen/Progestin Interventions." *JAMA* **273**: 199-208.

Thomas, C. V., M. L. Coker, et al. (1998). "Increased matrix metalloproteinase activity and selective upregulation in LV myocardium from patients with end-stage dilated cardiomyopathy." *Circulation* **97**(17): 1708-15.

Thompson, R. D., K. E. Noble, et al. (2001). "Platelet-endothelial cell adhesion molecule-1 (PECAM-1)-deficient mice demonstrate a transient and cytokine-specific role for PECAM-1 in leukocyte migration through the perivascular basement membrane." *Blood* **97**(6): 1854-60.

Toombs, C. F., S. McGee, et al. (1992). "Myocardial protective effects of adenosine. Infarct size reduction with pretreatment and continued receptor stimulation during ischemia." *Circulation* **86**(3): 986-94.

Tremblay, G. B., A. Tremblay, et al. (1997). "Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor beta." *Mol Endocrinol* **11**(3): 353-65.

Trial, J., R. D. Rossen, et al. (2004). "Inflammation and ischemia: macrophages activated by fibronectin fragments enhance the survival of injured cardiac myocytes." *Exp Biol Med (Maywood)* **229**(6): 538-45.

Tsai, M. J. and B. W. O'Malley (1994). "Molecular mechanisms of action of steroid/thyroid receptor superfamily members." *Annu Rev Biochem* **63**: 451-86.

Vaccarino, V., L. Parsons, et al. (1999). "Sex-based differences in early mortality after myocardial infarction. National Registry of Myocardial Infarction 2 Participants." *N Engl J Med* **341**(4): 217-25.

van der Schouw, Y. T., Y. van der Graaf, et al. (1996). "Age at menopause as a risk factor for cardiovascular mortality." *Lancet* **347**(9003): 714-8.

van Eeden, S. F., M. E. Klut, et al. (1999). "The use of flow cytometry to measure neutrophil function." *J Immunol Methods* **232**(1-2): 23-43.

van Eickels, M., R. D. Patten, et al. (2003). "17-beta-estradiol increases cardiac remodeling and mortality in mice with myocardial infarction." *J Am Coll Cardiol* **41**(11): 2084-92.

van Es, S. and P. N. Devreotes (1999). "Molecular basis of localized responses during chemotaxis in amoebae and leukocytes." *Cell Mol Life Sci* **55**(10): 1341-51.

Van Wart, H. E. and H. Birkedal-Hansen (1990). "The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family." *Proc Natl Acad Sci U S A* **87**(14): 5578-82.

Vanhoutte, D., M. Schellings, et al. (2005). "Relevance of matrix metalloproteinases and their inhibitors after myocardial infarction: A temporal and spatial window." *Cardiovasc Res.*

Vergely, C., V. Maupoil, et al. (2003). "Identification and quantification of free radicals during myocardial ischemia and reperfusion using electron paramagnetic resonance spectroscopy." *Arch Biochem Biophys* **420**(2): 209-16.

Vilatoba, M., C. Eckstein, et al. (2005). "17beta-estradiol differentially activates mitogen-activated protein-kinases and improves survival following reperfusion injury of reduced-size liver in mice." *Transplant Proc* **37**(1): 399-403.

Villarreal, F. J., M. Griffin, et al. (2003). "Early short-term treatment with doxycycline modulates postinfarction left ventricular remodeling." *Circulation* **108**(12): 1487-92.

Wagner, A. H., M. R. Schroeter, et al. (2001). "17beta-estradiol inhibition of NADPH oxidase expression in human endothelial cells." *Faseb J* **15**(12): 2121-30.

Wahl, P., C. Walden, et al. (1983). "Effect of estrogen/progestin potency on lipid/lipoprotein cholesterol." *N Engl J Med* **308**(15): 862-7.

Wakatsuki, A., Y. Okatani, et al. (2001). "Effect of medroxyprogesterone acetate on endothelium-dependent vasodilation in postmenopausal women receiving estrogen." *Circulation* **104**(15): 1773-8.

Wang, M., P. Crisostomo, et al. (2006). "Estrogen receptor-alpha mediates acute myocardial protection in females." *Am J Physiol Heart Circ Physiol* **290**(6): H2204-9.

Wang, P. and J. L. Zweier (1996). "Measurement of nitric oxide and peroxynitrite generation in the postischemic heart. Evidence for peroxynitrite-mediated reperfusion injury." *J Biol Chem* **271**(46): 29223-30.

Weber, K. T. (1989). "Cardiac interstitium in health and disease: the fibrillar collagen network." *J Am Coll Cardiol* **13**(7): 1637-52.

Wehling, M. (1997). "Specific, nongenomic actions of steroid hormones." *Annu Rev Physiol* **59**: 365-93.

Wehrens, X. H. and P. A. Doevendans (2004). "Cardiac rupture complicating myocardial infarction." *Int J Cardiol* **95**(2-3): 285-92.

Willenbrock, F. and G. Murphy (1994). "Structure-function relationships in the tissue inhibitors of metalloproteinases." *Am J Respir Crit Care Med* **150**(6 Pt 2): S165-70.

Williams, F. M., M. Kus, et al. (1994). "Effect of duration of ischaemia on reduction of myocardial infarct size by inhibition of neutrophil accumulation using an anti-CD18 monoclonal antibody." *Br J Pharmacol* **111**(4): 1123-8.

Williams, J. K., M. R. Adams, et al. (1992). "Short-term administration of estrogen and vascular responses of atherosclerotic coronary arteries." *J Am Coll Cardiol* **20**(2): 452-7.

Williams, P. W., R. Dyson, M. Bannister, L. (1989). Grays anatomy. Edinburgh, Churchill Livingstone.

Williamson, R. A., F. A. Marston, et al. (1990). "Disulphide bond assignment in human tissue inhibitor of metalloproteinases (TIMP)." *Biochem J* **268**(2): 267-74.

Wingrove, C. S., E. Garr, et al. (1998). "17beta-oestradiol enhances release of matrix metalloproteinase-2 from human vascular smooth muscle cells." *Biochim Biophys Acta* **1406**(2): 169-74.

Wright, D. G. and J. I. Gallin (1977). "A functional differentiation of human neutrophil granules: generation of C5a by a specific (secondary) granule product and inactivation of C5a by azurophil (primary) granule products." *J Immunol* **119**(3): 1068-76.

Wysocka, J., R. Lipartowska, et al. (2001). "[Granules of neutrophils]." *Postepy Hig Med Dosw* **55**(1): 177-88.

Xiaoquang Liu, T. H. C., Stanley M. Levenson, Marcos Rojkind (1997). "Wound fluids from saline solution-and Staphylococcus aureus peptidoglycan-innoculated sponges induce expression of matrix metalloproteinase 13 messenger ribonucleic acid by cultured rat fibroblasts." *Wound repair and regeneration* **5**: 348-354.

Xu, Y., I. A. Arenas, et al. (2003). "Estrogen modulation of left ventricular remodeling in the aged heart." *Cardiovasc Res* **57**(2): 388-94.

Yang, S. H., R. Liu, et al. (2004). "Mitochondrial localization of estrogen receptor beta." *Proc Natl Acad Sci U S A* **101**(12): 4130-5.

Yaron, G., A. Brill, et al. (2006). "C-reactive protein promotes platelet adhesion to endothelial cells: a potential pathway in atherothrombosis." *Br J Haematol* **134**(4): 426-31.

Ytrehus, K., Y. Liu, et al. (1994). "Rat and rabbit heart infarction: effects of anesthesia, perfusate, risk zone, and method of infarct sizing." *Am J Physiol* **267**(6 Pt 2): H2383-90.

Yu, H. P., T. Shimizu, et al. (2006). "Mechanism of cardioprotection following trauma-hemorrhagic shock by a selective estrogen receptor-beta agonist: up-regulation of cardiac heat shock factor-1 and heat shock proteins." *J Mol Cell Cardiol* **40**(1): 185-94.

Zak, R., A. F. Martin, et al. (1973). "Turnover of myocardial components in cardiac hypertrophy." *Recent Adv Stud Cardiac Struct Metab* **3**: 603-14.

Zhai, P., T. E. Eurell, et al. (2000). "Effect of estrogen on global myocardial ischemia-reperfusion injury in female rats." *Am J Physiol Heart Circ Physiol* **279**(6): H2766-75.

Zhai, P., T. E. Eurell, et al. (2000). "Myocardial ischemia-reperfusion injury in estrogen receptor-alpha knockout and wild-type mice." *Am J Physiol Heart Circ Physiol* **278**(5): H1640-7.

Zouki, C., B. Haas, et al. (2001). "Loss of pentameric symmetry of C-reactive protein is associated with promotion of neutrophil-endothelial cell adhesion." *J Immunol* **167**(9): 5355-61.

Zweier, J. L. (1988). "Measurement of superoxide-derived free radicals in the reperfused heart. Evidence for a free radical mechanism of reperfusion injury." *J Biol Chem* **263**(3): 1353-7.

Zweier, J. L., P. Kuppusamy, et al. (1989). "Measurement and characterization of postischemic free radical generation in the isolated perfused heart." *J Biol Chem* **264**(32): 18890-5.